

Application No. 10/018,396

Reply to Office Action

*REMARKS/ARGUMENTS**Examiner Interview*

Applicant thanks Examiner Ungar for the courtesy extended to their representatives, John L. Gase, during the telephonic interview held on April 28, 2006. The matters discussed during the interview are substantially as set forth herein.

*Request for Interview with Examiner and Supervisory Patent Examiner*

Applicant hereby requests a telephonic interview with the Examiner and the Supervisory Patent Examiner in charge prior to the issuance of an office action on the merits. The Examiner is invited to contact the undersigned attorney to arrange a convenient time for the interview.

*The Pending Claims*

Claims 1-8 are currently pending. Claims 1-8 are directed to a method of diagnosing cancer in a patient.

*The Office Action*

The Office maintains and makes final the previous rejections of claims 1-8 as allegedly lacking enablement and written description under 35 U.S.C. § 112, first paragraph and as indefinite under 35 U.S.C. § 112, second paragraph.

*Response to Final Office Action and Advisory Action*

A response to the Final Office Action was filed on January 3, 2006 pursuant to the expedited examining procedure of 37 C.F.R. § 1.116 (i.e., within 2-months of the mailing of the Final Office Action). An advisory action was not mailed prior to the expiration of the 6-month statutory period, necessitating the filing of a Request for Continued Examination (RCE) and the present Response to Office Action.

Although the advisory action was not mailed prior to the end of the 6-month statutory procedure, the Examiner communicated an advisory action by facsimile (without a mailing date) to Applicant one business day prior to the end of the statutory period. As the advisory action has not been mailed as of the date of this submission, a copy of the advisory action is included herewith to complete the record.

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The advisory action indicates that the Applicant's prior arguments were not persuasive, and that none of the prior rejections had been withdrawn. The advisory action also reaffirms the Office's earlier acknowledgement that certain subject matter would be allowable, as discussed below. Finally, during the aforementioned interview, the Examiner indicated that certain evidence that accompanied the Response to Final Office Action was not considered as it was not timely submitted.

Accordingly, Applicants' submit the following comments and accompanying evidence, including the Declaration of Yoon S. Cho-Chung, M.D., Ph.D. dated May 1, 2006 for full consideration by the Office.

*Discussion of the Non-Enablement Rejection*

The Office maintains its prior rejection of the pending claims as non-enabled in three respects: (a) with respect to the use of extra-cellular PKA protein levels (as opposed to enzyme activity levels) to diagnose cancer because the specification allegedly fails to provide any method for distinguishing extra-cellular PKA from membrane bound or intracellular PKA, and fails to provide a nexus between overexpression of extra-cellular PKA and increased enzyme activity, (b) with respect to the use of *any* patient sample because the Office alleges that the use of a non-fluid patient sample is not enabled, and (c) with respect to the diagnosis of *any* cancer because the Applicant allegedly has failed to provide ample proof of its effectiveness with respect to sarcomas.

With respect to the non-enablement rejections of record, the Office has acknowledged that a method of diagnosing cancer in a patient comprising assaying the protein level of extra-cellular PKA in serum using an antibody to the catalytic subunit of PKA would be acceptable. This is inconsistent with the Office's arguments that the application fails to provide a method for distinguishing extra-cellular PKA from other forms of PKA, or fails to provide a nexus between extra-cellular PKA enzyme activity levels and extra-cellular PKA protein levels (ground (a) above). Nevertheless, the Office has made clear in the advisory action that all previously stated grounds of the non-enablement rejection still apply. Accordingly, each basis of the non-enablement rejection is addressed below.

In summary of Applicant's position, the Office has not presented sufficient evidence to support its allegations and has, thus, failed to establish a *prima facie* case of non-enablement under Section 112. Furthermore, the Office has failed to give proper

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consideration to the evidence and arguments presented by Applicant, which overcome the rejections. For these reasons, the non-enablement rejections are improper and should be withdrawn.

*A. The Office Bears the Burden of Proof*

The manner and process of making and using Applicant's invention, as disclosed in the application, must be taken as being in compliance with the enablement requirement of Section 112 unless there is a reason to doubt the objective truth of the statements contained therein. *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971). Furthermore, "it is incumbent upon the Patent Office ... to explain why it doubts the truth or accuracy of any statement in a supporting disclosure *and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement.*" *In re Marzocchi*, 439 F.2d at 224, 169 USPQ at 370 (emphasis added). A general allegation of "unpredictability in the art" is not a sufficient reason to support a rejection for lack of adequate written description. *Id.* See also, MPEP § 2163.04.

*B. Assaying Extra-cellular PKA Protein Level*

The Office alleges that the specification does not enable one of ordinary skill in the art to assay extra-cellular PKA protein level as a basis for the diagnosis of cancer for two reasons: (1) the specification allegedly does not provide any way to distinguish extra-cellular PKA from intra-cellular or membrane-bound (ecto-cellular) PKA, and (2) the specification allegedly fails to provide sufficient proof that extra-cellular PKA protein levels (as opposed to enzyme activity levels) are elevated in cancer.

With respect to reason (1), Applicant previously argued that extra-cellular PKA is distinguished from intra-cellular and ecto-cellular PKA on the basis that it is present outside of the cell, hence the name "extra-cellular PKA." In response, the Final Office Action states that Applicant is arguing limitations that are not in the claims (Final Office Action at p. 2-3). The Office further argues that one of ordinary skill in the art would not be able to distinguish between extra-cellular PKA and intra-cellular or ecto-cellular PKA released "from the inevitably lysed cells in the sample which, because of endogenous cAMP, would be expected to include active catalytic subunits" (Final Office Action at p. 3).

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As to the allegation that Applicant is arguing limitations not in the claims, the Office is incorrect. The claims are explicitly recite assaying "extra-cellular" PKA. One of ordinary skill in the art would recognize that "extra-cellular" PKA is to be found outside of the cell. Furthermore, the specification states that "cell-lysis should be kept to a minimum so that determination of PKA accurately reflects the amount of extra-cellular PKA, not intracellular PKA" (specification at p. 9). As read by one of ordinary skill in the art, this instruction reinforces that the claims require assaying PKA levels outside of the cell. Thus, contrary to the Office's assertion, Applicant is arguing limitations that are expressly recited in the claims.

As to the Office's argument that "inevitable" cell lysis in a sample would prevent one of ordinary skill in the art from assaying the protein level of extra-cellular PKA, the Office's reasoning is legally insufficient and factually incorrect.

First, the Office provides *no evidence* that any significant amount of contamination by cell-lysis is "inevitable" when practicing the claimed invention, or that one of ordinary skill in the art would not possess the requisite skill or knowledge required to address such contamination without undue experimentation. In the advisory action, the Office cites to page 9 of the specification as evidence of its position. However, as discussed briefly above, page 9 of the specification merely indicates that the extent of cell lysis should be kept to a minimum. In no way does such a statement suggest that a significant amount of contamination by cell lysis is inevitable, or that one of ordinary skill in the art would not be able to address such contamination. It is the Office's burden to come forward with evidence supporting the non-enablement rejection. In the absence of such evidence, the burden does not shift to the applicant to prove that the invention works as it is described. Because the Office has failed to support its allegations with any proof, the stated basis for the rejection is legally insufficient to establish a *prima facie* case under Section 112.

Second, the reasoning is factually erroneous in that (a) methods of detecting contamination by cell lysis are well-known and demonstrated in the application, (b) significant cell lysis or degradation is not "inevitable," and (c) one of ordinary skill in the art *can* determine whether the PKA protein level of a sample is due to extra-cellular PKA as opposed to other forms of PKA based on the teachings of the application. It is well-known in the art that LDH levels in a sample can be used to determine whether any appreciable amount of cell lysis has occurred, which method is demonstrated in most of the Examples (e.g., Examples 1 and 8). Furthermore, Example 8 demonstrates by LDH levels that the serum

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samples used therein did not show signs of significant cell degradation. Thus, significant cell lysis is not inevitable. Also, to confirm that a given protein level corresponds to extra-cellular as opposed to intra-cellular or ecto-cellular PKA, one of ordinary skill in the art can introduce cAMP into the sample and measure a change in the enzyme activity level, as demonstrated in several of the Examples (e.g., Examples 1 and 8). Since extra-cellular PKA exists in the free catalytic form, it cannot be activated by cAMP, thereby distinguishing extra-cellular PKA from the other forms.

The Office implies that distinguishing extra-cellular PKA from the other forms of PKA on the basis of cAMP activation would not be possible when contamination by cell lysis is present due to the presence of "endogenous cAMP." Again, the Office fails to provide any evidence supporting this hypothesis. Furthermore, it appears that Office's hypothesis is incorrect. As demonstrated by the Examples, extra-cellular PKA is not activated by cAMP, but intracellular PKA from lysed-cell extracts is activated by cAMP (e.g., Example 1). Thus, contrary to the Office's hypothesis, the endogenous cAMP allegedly present in a lysed cell sample does not prevent one from distinguishing between extra-cellular and other forms of PKA on the basis of cAMP activation.

The Office further states in the advisory action that the foregoing arguments are not persuasive because "an activated catalytic cell unit, regardless of how it is activated, is still activated." The Office's response misses the arguments completely. The examples show that the intracellular and membrane-bound PKA in a lysed cell sample can be activated, but extra-cellular PKA cannot (extracellular cAMP is already active). Thus, if cAMP is added to a given sample and the PKA activity increases, the sample contains intracellular and/or ecto-cellular PKA. By way of contrast, if no substantial increase in the PKA activity of sample is observed upon addition of cAMP, the sample is relatively free of intracellular or ecto-cellular PKA, and any PKA activity is attributable to extra-cellular PKA. Thus, one of ordinary skill in the art following the teachings of the present application can distinguish between extra-cellular PKA and other forms of PKA based on cAMP activation.

With respect to reason (2), above, the Office bases its position entirely on speculation as to what mechanisms, other than elevated PKA expression, might underlie Applicant's invention. In setting forth the rejection in the prior Office Action, the Office references various factors that affect the enzyme activity of proteins other than PKA, and concludes that PKA *might* behave similarly to such other proteins:

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Although Applicant hypothesizes that exemplified increased activity of extra-cellular cAMP in serum of carcinoma cancer patients is due to increased expression of one or both of the subunits of [extra-cellular PKA], given the above, *it is just as reasonable to hypothesize* that the increased activity demonstrated in the serum of cancer patients is due to alteration of sites associated with allosteric effectors, reduced availability of inhibitor, reduced affinity for inhibitor, mutation of the catalytic subunit that leads to constitutive activation, altered affinity for regulatory subunit, altered affinity for substrate.

(Office Action dated June 2, 2005 at p. 9). However, the Office presents no *evidence* that PKA is influenced by any of the factors mentioned. Furthermore, the Examiner has not been qualified on record as an expert in the field, and her hypothesis as to what mechanisms might or might not be involved based on reports regarding different proteins cannot properly be relied upon as evidence. Thus, the Office has not provided any evidence that Applicant's disclosure is incorrect. In the absence of such evidence, Applicant's disclosure is presumed to be correct by law (see Section A, above).

Moreover, the Office completely ignores substantial evidence presented in the application itself that shows extra-cellular PKA activity is linked to increased expression. For instance, Example 1 of the application shows that extra-cellular PKA is shed or secreted from cancer cells into a culture medium. This evidence alone would lead one of ordinary skill in the art to expect elevated serum extra-cellular PKA activity to be caused, at least in part, by increased extra-cellular PKA protein levels in serum. Furthermore, Example 5 of the application shows that increased extra-cellular PKA activity is tied to increased intracellular expression of PKA, providing further evidence that the observed increase in serum extra-cellular PKA activity is due to an increased level of extra-cellular PKA protein as opposed to some other factor. This evidence is in agreement with earlier observations that cellular PKA expression levels in tumors is increased (specification at p. 3, lines 10-15).

While the evidence presented in the application itself is sufficient to overcome the rejection, more recent publications further confirm the impropriety of the rejection. For instance, the inventors have shown in other publications that autoantibodies to extra-cellular PKA can be used as a basis to detect cancer (WO 2005/088312 at pp. 19-23 (copy enclosed)). Since autoantibodies are generated in response to the introduction of an antigen into the serum, these results demonstrate that extra-cellular PKA serum protein levels are enhanced in cancer patients. Also, Mani et al. reports that extra-

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cellular PKA protein levels in the serum of cancer patients have been used as a basis to track patient responses to various cancer treatments in clinical trials (see, e.g., Mani et al., Ann. N.Y. Acad. Sci., 1002, 252-262 at 257 (2003) (copy enclosed)).

For the foregoing reasons, the Office has failed to present sufficient proof to establish *prima facie* non-enablement with respect to the use of extra-cellular PKA protein levels to diagnose cancer. Furthermore, the evidence presented by Applicant is sufficient to overcome any such rejection. Accordingly, the non-enablement rejection with respect to these grounds should be withdrawn.

*B. Use of a Non-Fluid Patient Sample*

The Office argues that the claims do not enable the use of a sample other than a fluid sample because the application provides no way to distinguish between extra-cellular PKA and other forms of PKA using a solid (e.g., non-fluid) sample from a patient. Applicant argued in her prior response that a solid sample from a patient can be used, for instance, by culturing the sample in a medium and assaying the extra-cellular fraction of the cultured sample, as demonstrated in the Examples of the application. In response, the Office states that Applicant's arguments are not persuasive because no nexus between elevated protein level and elevated enzyme activity level has been established (Final Office Action at p. 8).

The Office's response does not address Applicant's argument. By re-directing the argument regarding the nature of the sample to the "nexus" between enzyme activity level and protein level, the Office addresses only whether protein levels can be used at all (see Section B, above), not whether the application enables the use of a solid sample from a patient. In fact, the Office provides no reasoning or evidence whatsoever supporting its assertion that a solid sample could not be used.

In the advisory action, the Office asserts that it has not re-directed the argument. However, no further reasoning in support of the rejection is provided.

For the foregoing reasons, the Office has failed to present sufficient proof to establish *prima facie* non-enablement with respect to the use of a solid patient sample in conjunction with the claimed method. Furthermore, the evidence presented by Applicant is sufficient to overcome any such rejection. Accordingly, the non-enablement rejection with respect to these grounds should be withdrawn.

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*C. Diagnosis of Cancer in a Patient with Sarcoma*

The Office maintains its prior non-enablement rejection of the claims on the basis that Applicant's have submitted no proof that the claimed method would be effective for diagnosing cancer in a patient with a sarcoma. In Applicant's prior response to office action, a declaration was submitted introducing evidence that extra-cellular PKA levels were elevated in sarcoma cell lines. The Office has responded that the evidence is insufficient because (a) the Examiner has no way of knowing whether the cell lines were engineered to constitutively express extra-cellular PKA, (b) the "normal" cell lines are not defined and the Examiner cannot, therefore, properly assess the evidence, and (c) the extra-cellular PKA levels of the normal sample are higher than the level of a "control" defined in certain dependent claims. The Office's reasoning is insufficient to maintain the rejection.

First, as a general matter, the non-enablement rejection is not supported by sufficient evidence. In the prior Office Action setting forth the rejection, the Office cites *Taber's Cyclopedic Medical Dictionary* and *Molecular Biology of the Cell* for the general proposition that sarcomas and carcinomas are different classifications of cancer originating from different types of tissues (Office Action dated June 2, 2005 at pp. 12-13). The Office also cites *Immunology Today* for the general proposition that there is heterogeneity in tumors not only between patients but even between metastatic sites within a single patient (Office Action dated June 2, 2005 at pp. 12-13). From these general propositions that "all cancers are different," the Office concludes that extra-cellular PKA would not be expected to serve as a marker for sarcomas.

However, the general proposition that "all cancers are different" does not refute the use of extra-cellular PKA as a biomarker for all cancers including sarcoma. The Office acknowledges that extra-cellular PKA can be used as a biomarker across a broad range of cancers and even remarks that "the finding that the same marker is found across a broad range of epithelial cancers is *surprising and unexpected*" (Office Action dated June 2, 2005 at p. 12). The evidence that extra-cellular PKA can be used as a marker for a broad range of cancers, despite the proposition that all cancers are different, strongly suggests that it *can* be used to diagnose sarcomas. In view of the specific evidence in the application that extra-cellular PKA is a biomarker for a broad range of cancers, the generic evidence presented by the Office is insufficient to establish a *prima facie* case of non-enablement.



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Furthermore, even if the Office adequately supported the rejection, which it did not, the evidence submitted by way of declaration in the Applicant's prior response is more than adequate to overcome the non-enablement rejection, and the Office has not provided sufficient reasons for rejecting the evidence.

With respect to reason (a), the Examiner has absolutely no reason to believe the cell-lines were engineered to constitutively express extra-cellular PKA. The declaration and experimental data was submitted as evidence that that claimed method could be used to diagnose sarcoma. Cell-lines that had been engineered to constitutively express extra-cellular PKA would not be useful for such a purpose. Indeed, it is wholly improper for the Examiner to imply that Applicant would submit evidence that would mislead the Office. Nevertheless, to clarify the record, the cells used in the reported experiments were not engineered to constitutively express extra-cellular PKA, as per the Declaration of Yoon S. Cho-Chung, M.D., Ph.D. dated May 1, 2006 (submitted herewith).

With respect to reason (b), the exclusion of the specific identity of the "normal" cells used does not diminish the evidentiary weight of the declaration. The Examiner has no reason to believe that an inappropriate type of "normal" cell was used except, again, to the extent the Examiner believes Applicant wishes to mislead the Office. Furthermore, the Examiner has not been established on record as an expert in the field, and cannot properly judge whether a given cell type is an appropriate "normal" cell in the context of the inventor's experiments. The Applicant's declaration must be taken as true and correct on its face. Nevertheless, in the interest of furthering the prosecution of the application, the "normal" cells were non-cancerous human prostate (PrEC 5500) and breast (MCF10A) cells selected by the inventors as appropriate controls in the context of the reported experiments, as per the Declaration of Yoon S. Cho-Chung, M.D., Ph.D. dated May 1, 2006 (submitted herewith).

With respect to reason (c), the Office's comparison of the experimental data to dependent claims 3 and 6 is misplaced. Dependent claims 3 and 6 explicitly refer to the level of enzyme activity in a control sample of serum or urine. The experimental data submitted by declaration pertains to cultured cell samples and, therefore, is not properly comparable to control samples of serum or urine. Nevertheless, the experimental data shows that the cultured sarcoma cell samples exhibited extra-cellular PKA levels higher than the experimental control and, thus, supports the claims. Experimental evidence need not directly

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support all of the claims in order to be probative evidence of the ability to use the claimed method to diagnose cancer, including sarcoma.

Although the foregoing is sufficient to overcome the rejection, more recent publications further confirm the impropriety of the rejection. For instance, the inventors have shown that serum extra-cellular PKA protein levels are increased in a broad range of cancers including sarcoma (WO 2005/088312 (copy enclosed)). Also, extra-cellular PKA serum levels have been used as a basis to track the response of cancer patients, including sarcoma patients, in clinical trials (see, e.g., Lockhart et al., *Proc. Am. Soc. Clin. Oncol.*, 22, 245 (2003) (copy enclosed)). The above references were published after the filing date of the present application; however, they rely upon the teachings of the application, and provide proof that the teachings of the application are true. For example, the disclosure of the above '312 PCT application confirms that the increase in extra cellular PKA levels in the serum of cancer patients is due to an increase in protein expression, as opposed to the other factors that were proposed by the Office. Also, the disclosure of Lockhart et al. confirms that the invention can be used as to all cancers, including sarcomas, contrary to the Office's arguments. Thus, the above references are relied upon not as a supplement of the disclosure of the application as filed, but, rather, as evidence that the application as filed enables the full scope of the claimed subject matter.

For the foregoing reasons, the Office has failed to present sufficient proof to establish *prima facie* non-enablement with respect to the use of the claimed method to diagnose cancer in a patient with sarcoma. Furthermore, the evidence presented by Applicant is sufficient to overcome any such rejection. Accordingly, the non-enablement rejection with respect to these grounds should be withdrawn.

#### *Discussion of the Written Description Rejection*

The Office maintains its prior rejection of the pending claims as lacking adequate written description under 35 U.S.C. § 112, first paragraph. In particular, the Office alleges that Applicant attempts to claim a novel protein (e.g., extra-cellular PKA) by its function without providing a description of the structure of the protein.

During the aforementioned interview, the Examiner indicated that she had reconsidered her position as to written description, and that she believed extra-cellular PKA was similar or identical to the catalytic subunit of PKA. The Examiner also indicated that

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certain claims directed to the use of extra-cellular PKA protein and enzyme activity levels would be acceptable, implying that the written description rejection of the claims would not be maintained. Nevertheless, in the interest of clarifying the record, Applicant addresses the rejection below.

Applicant previously argued that the claims are not directed to a novel protein *per se*, and that the application nevertheless provides an adequate description of the structure of extra-cellular PKA such that one of ordinary skill in the art would recognize that the inventors were in possession of the claimed method of diagnosing cancer. In particular, the application demonstrates that extra-cellular PKA is similar or identical to the free catalytic subunit of PKA, which is a well-characterized protein. The Final Office Action, however, argues that these reasons are not persuasive because the application states that an antibody can be used to distinguish between extra-cellular PKA and other forms of PKA. Thus, the Office concludes that the two proteins are different, but that the differences are not described.

The statement that an antibody can be used to distinguish between extra-cellular PKA and other forms of PKA is not inconsistent with the similarity or identity between extra-cellular PKA and the catalytic subunit of other forms of PKA. For instance, an antibody to the regulatory "R" subunit of intracellular PKA could be used to bind to intracellular PKA, which comprises a catalytic "C" subunit and a regulatory "R" subunit, but would not bind to extra-cellular PKA, which exists as a "free" C subunit. Thus, such an antibody could be used to distinguish between extra-cellular PKA and intra-cellular PKA. Accordingly, the Office's stated reasoning does not support the written description rejection.

As with the enablement requirement, the Office bears the burden of showing non-compliance with the written description requirement of Section 112. There is a strong presumption that an adequate written description of the claimed invention is present when the application is filed. *In re Wertheim*, 541 F.2d 257, 263, 191 U.S.P.Q. 90, 97 (C.C.P.A. 1976). The Office has not presented sufficient evidence or reasoning to show that the claims lack adequate written description support, and has, thus, failed to establish a *prima facie* case under Section 112. Accordingly, the rejection is improper and should be withdrawn.

#### *Discussion of the Indefiniteness Rejection*

The Office rejects the claims as indefinite for "recitation of ECPKA as the sole means of identifying the protein product to be assayed" (Office Action dated June 2, 2005 at p. 16).

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The Office refers to "ECPKA" as a laboratory designation, and argues that different laboratories may use the same laboratory designations to define completely different proteins.

In her prior response, Applicant informed the Office that, as recited in claim 1, "ECPKA" is an abbreviation that stands for "extra-cellular cAMP dependent protein kinase." The claim term, as read in conjunction with the specification, is neither vague nor indefinite. It is inapposite whether or not the term "ECPKA" also is used as a laboratory designation because the claims expressly refer to "extra-cellular cAMP dependent protein kinase," which is not a laboratory designation but, rather, a description of the protein and its location outside of the cell.

The Final Office Action responds to Applicants reasoning by stating "[Applicant's] argument has been considered but has not been found persuasive because surprisingly discovered PKA is not defined by the specification or claims for the reasons of record and for the reasons set forth above" (Final Office Action at p. 10). By "reasons set forth above," Applicant can only assume that the Office is referring to the reasons underlying the enablement rejection or the written description rejection (or both). In either case, the legal standards for enablement and written description under Section 112, first paragraph, are not the proper standards upon which to base an indefiniteness rejection under Section 112, second paragraph.

Thus, the Final Office Action neither addresses Applicant's arguments nor further explains its position with respect to the indefiniteness rejection. Accordingly, Applicant reiterates her prior position that the claims are clear and definite within the meaning of Section 112, second paragraph, for the reasons already of record, and the rejection of the claims as indefinite should be withdrawn.

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*Conclusion*

If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,



John L. Gase, Reg. No. 47,590  
LEYDIG, VOIT & MAYER, LTD.  
Two Prudential Plaza, Suite 4900  
180 North Stetson Avenue  
Chicago, Illinois 60601-6780  
(312) 616-5600 (telephone)  
(312) 616-5700 (facsimile)

Date: May 1, 2006

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an L. Gase COMPANY:To: EM2  
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37 C.F.R. 1.6 sets forth the types of correspondence that can be communicated to the Patent and Trademark Office via facsimile transmissions. Applicants are advised to use the certificate of facsimile transmission procedures when submitting a reply to a non-final or final Office action by facsimile (37 CFR 1.8(a)).

**Fax Notes:**

Attached is a copy of the Advisory Action that will be mailed to you for your review since, unfortunately, the signed action will not be mailed before Monday. If you have any questions or comments you can reach me at 571-272-0837  
Susan Ungar  
Primary Patent Examiner

Date and time of transmission: Friday, April 28, 2006 10:12:02 AM  
Number of pages including this cover sheet: 04

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<b>Advisory Action Before the Filing of an Appeal Brief</b>	<b>Application No.</b> 10,018,396	<b>Applicant(s)</b> CHO-CHUNG, YOON S.
	<b>Examiner</b> Susan Ungar	<b>Art Unit</b> 1642

**-The MAILING DATE of this communication appears on the cover sheet with the correspondence address -**

THE REPLY FILED 09 January 2006 FAILS TO PLACE THIS APPLICATION IN CONDITION FOR ALLOWANCE.

1. ☒ The reply was filed after a final rejection, but prior to or on the same day as filing a Notice of Appeal. To avoid abandonment of this application, applicant must timely file one of the following replies: (1) an amendment, affidavit, or other evidence, which places the application in condition for allowance; (2) a Notice of Appeal (with appeal fee) in compliance with 37 CFR 41.31; or (3) a Request for Continued Examination (RCE) in compliance with 37 CFR 1.114. The reply must be filed within one of the following time periods:

a) ☒ The period for reply expires 3 months from the mailing date of the final rejection.

b) ☐ The period for reply expires on: (1) the mailing date of this Advisory Action, or (2) the date set forth in the final rejection, whichever is later. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of the final rejection.

Examiner Note: If box 1 is checked, check either box (a) or (b). ONLY CHECK BOX (b) WHEN THE FIRST REPLY WAS FILED WITHIN TWO MONTHS OF THE FINAL REJECTION. See MPEP 706.07(f).

Extensions of time may be obtained under 37 CFR 1.136(a). The date on which the petition under 37 CFR 1.136(a) and the appropriate extension fee have been filed is the date for purposes of determining the period of extension and the corresponding amount of the fee. The appropriate extension fee under 37 CFR 1.17(a) is calculated from: (1) the expiration date of the shortened statutory period for reply originally set in the final Office action; or (2) as set forth in (b) above, if checked. Any reply received by the Office later than three months after the mailing date of the final rejection, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**NOTICE OF APPEAL**

2. ☐ The Notice of Appeal was filed on \_\_\_\_\_. A brief in compliance with 37 CFR 41.37 must be filed within two months of the date of filing the Notice of Appeal (37 CFR 41.37(a)), or any extension thereof (37 CFR 41.37(e)), to avoid dismissal of the appeal. Since a Notice of Appeal has been filed, any reply must be filed within the time period set forth in 37 CFR 41.37(a).

**AMENDMENTS**

3. ☐ The proposed amendment(s) filed after a final rejection, but prior to the date of filing a brief, will not be entered because

(a) ☐ They raise new issues that would require further consideration and/or search (see NOTE below);

(b) ☐ They raise the issue of new matter (see NOTE below);

(c) ☐ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal; and/or

(d) ☐ They present additional claims without canceling a corresponding number of finally rejected claims.

NOTE: \_\_\_\_\_. (See 37 CFR 1.116 and 41.33(a)).

4. ☐ The amendments are not in compliance with 37 CFR 1.121. See attached Notice of Non-Compliant Amendment (PTOL-324).

5. ☐ Applicant's reply has overcome the following rejection(s): \_\_\_\_\_.

6. ☐ Newly proposed or amended claim(s) \_\_\_\_\_ would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claim(s).

7. ☒ For purposes of appeal, the proposed amendment(s): a) ☐ will not be entered, or b) ☒ will be entered and an explanation of how the new or amended claims would be rejected is provided below or appended.

The status of the claim(s) is (or will be) as follows:

Claim(s) allowed: none.

Claim(s) objected to: none.

Claim(s) rejected: 1-8.

Claim(s) withdrawn from consideration: none.

**AFFIDAVIT OR OTHER EVIDENCE**

8. ☒ The affidavit or other evidence filed after a final action, but before or on the date of filing a Notice of Appeal will not be entered because applicant failed to provide a showing of good and sufficient reasons why the affidavit or other evidence is necessary and was not earlier presented. See 37 CFR 1.116(e).

9. ☐ The affidavit or other evidence filed after the date of filing a Notice of Appeal, but prior to the date of filing a brief, will not be entered because the affidavit or other evidence failed to overcome all rejections under appeal and/or appellant fails to provide a showing a good and sufficient reasons why it is necessary and was not earlier presented. See 37 CFR 41.33(d)(1).

10. ☐ The affidavit or other evidence is entered. An explanation of the status of the claims after entry is below or attached.

**REQUEST FOR RECONSIDERATION/OTHER**

11. ☒ The request for reconsideration has been considered but does NOT place the application in condition for allowance because:  
See Continuation Sheet.

12. ☐ Note the attached Information Disclosure Statement(s). (PTO/SB/08 or PTO-1449) Paper No(s). \_\_\_\_\_

13. ☐ Other: \_\_\_\_\_

**LAB**  
Susan Ungar  
Primary Examiner  
Art Unit 1642

USPTO 4/28/2006 10:12 AM PAGE 3/004 Fax Server  
: John L. Gase COMPANY:

Continuation Sheet (PTO-303)

Application No. 10/018,396

Continuation of 11. does NOT place the application in condition for allowance because: 1. Claims 1-8 remain rejected under 35 USC 112, first paragraph for the reasons previously set forth in the paper mailed November 1, 2005, Section 4, pages 2-9.

Applicant argues that at the interview of December 20, 2005 Examiner made reference to a potential rejection of the claims on the basis that enzyme activity of PKA cannot be detected in urine and that the new grounds of rejection were not made of record. If the office wishes to reject the claims on new grounds, a non-final office action should be issued.

The argument has been considered but has not been found persuasive since Applicant is correct, there is no rejection of record drawn to PKA activity in urine. Examiner was simply discussing possible allowable material with Applicant's attorney and asking if PKA activity could be detected in urine in an effort to provide customer service as well as to advance prosecution. All of the claims are properly rejected for the reasons of record and no additional rejections are necessary to complete the rejections of the claims as currently constituted. Examiner apologizes for any inconvenience or misunderstanding and will be careful in the future to only discuss, with Applicant's attorney, allowable material drawn to specific limitations claimed and discussed in written office actions.

Applicant further states that since Examiner has indicated that claims directed to a method of diagnosing carcinoma in a patient comprising assaying the protein level of intracellular PKA in a body fluid using an antibody to the catalytic subunit of extracellular PKA would be acceptable, thus, Applicant understands that the Office no longer holds its former position that the application fails to provide a method for distinguishing extracellular PKA from other forms of PKA. The argument has been considered but has not been found persuasive because Applicant's statements are incorrect and again mischaracterize Examiner's position. The rejections of record stand for the reasons of record. The claims are not drawn to a method for distinguishing extracellular PKA from other forms of PKA, the claims are drawn to a method of diagnosing all forms of cancer in a patient comprising assaying any sample from said patient. Thus, Applicant appears to be arguing limitations not recited in the claims as currently constituted. In the interests of compact prosecution Examiner made clear, on the record, claim language that might be allowable in this Application. Examiner again apologizes for any inconvenience or misunderstanding and will be careful in the future to only discuss, with Applicant's attorney, allowable material drawn to specific limitations claimed and discussed in written office actions.

Applicant reiterates arguments drawn to the localization of extracellular PKA and states that no evidence is provided of significant contamination. The argument has been considered but has not been found persuasive given the teachings of the specification on page 9 of the specification.

Applicant argues that extra-cellular PKA is not activated by cAMP but intracellular PKA is activated by cAMP, thus the endogenous cAMP does not prevent one from distinguishing between extra-cellular and other forms of PKA on the basis of cAMP activation. The argument has been considered but has not been found persuasive as an activated catalytic cell unit, regardless of how it is activated would still be activated.

Applicant argues that Examiner presents no evidence in support of her hypothesis in response to Applicant's hypothesis. The argument has been considered but has not been found persuasive since Examiner never stated that the hypothesis was correct, only that it was as reasonable as Applicant's.

Applicant reiterates arguments that show that extra-cellular PKA activity is linked to increased expression in cancer cell lines and argues again that PKA expression is shed into culture medium and thus one would expect elevated serum extra-cellular PKA in serum. The argument has previously been considered but has not been found persuasive because the claims are not drawn to elevated serum extra-cellular PKA activity.

It is noted that Applicant has not addressed issues raised in the Final Rejection drawn to distinguishing membrane bound from ECPKA, does not address inability to assay ECPKA by determining the level of the R subunit, does not address lack of nexus between PKA activity and level, does not address the identity of the catalytic subunit of PKA/EctoPKA, does not address the teachings of Weber et al., does not address controls for ECPKA activity, does not address the apparent discrepancy between activity of normal controls and the instantly claimed activity level for controls, all discussed in pages 3-7 of the Final Rejection.

Applicant reiterates arguments drawn to non-fluid patient samples and argues that Examiner has redirected the argument by responding to the argument that no nexus has been established between activity level and the presence of elevated levels of ECPKA. The argument has been considered but has not been found persuasive because a review of the record reveals that Applicant again is incorrect and is mischaracterizing Examiner's response. Examiner did not redirect the argument and specifically and correctly responded to Applicant's argument drawn to fractionating solid samples where Applicant specifically argued that "...the example disclose culturing cells in a medium and testing the medium.... For PKA activity, thereby providing specific guidance." Applicant is clearly mischaracterizing both Examiner's response and Applicant's previous arguments.

Applicant argues that the references cited by Examiner drawn to different cancer types is not sufficient and that the evidence of ECPKA in a broad range of cancers is sufficient to enable the finding for all cancers and the evidence submitted in the Cho Declaration is sufficient to overcome the grounds of rejection. It is noted that Applicant's statements attempting to perfect the Cho Declaration, in the absence of a Declaration stating the facts, are not persuasive. The argument has been considered but has not been found persuasive for the reasons of record, and the Cho Declaration, drawn to cell line activity, is not persuasive for the reasons of record.

The arguments have been considered but have not been found persuasive and the rejection is maintained.

2. Claims 1-8 remain rejected under 35 USC 112, first paragraph for the reasons previously set forth in the paper mailed November 1, 2005, Section 5, pages 9-10.

Applicant states that Examiner indicated in the interview of December 20, 2005 that she had reconsidered her position as to written description and that she believed extra-cellular PKA was similar or identical to the catalytic subunit of PKA and that claims directed to the use of ECPKA protein and enzyme levels would be acceptable, implying that the written description rejection of the claims would not be maintained. Applicant's statement is again misleading and incorrect. Examiner never indicated that she had reconsidered her position as to written description or that claims directed to the use of ECPKA protein and enzyme levels would be acceptable. It is suggested that Applicant review the interview summary for said interview as well as Applicant's discussion of said interview where Applicant was clearly aware that what Examiner stated appeared to be allowable was the assay of body fluid for protein with an antibody to the catalytic domain of PKA. Examiner specifically put on the record that claims drawn to a method of diagnosing carcinoma in a patient comprising assaying the protein level of PKA in a body fluid using an antibody to the catalytic subunit of extracellular PKA would be acceptable, thus, Applicant understands that the Office no longer holds its former position that the application fails to provide a method for distinguishing extracellular PKA from other forms of PKA. The argument has been considered but has not been found persuasive because Applicant's statements are incorrect and again mischaracterize Examiner's position. The rejections of record stand for the reasons of record. The claims are not drawn to a method for distinguishing extracellular PKA from other forms of PKA, the claims are drawn to a method of diagnosing all forms of cancer in a patient comprising assaying any sample from said patient. Thus, Applicant appears to be arguing limitations not recited in the claims as currently constituted. In the interests of compact prosecution Examiner made clear, on the record, claim language that might be allowable in this Application. Examiner again apologizes for any inconvenience or misunderstanding and will be careful in the future to only discuss, with Applicant's attorney, allowable material drawn to specific limitations claimed and discussed in written office actions.



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## Continuation Sheet (PTO-303)

Application No. 10/018,386

is immunologically and biochemically identical to PKA catalytic subunit C alpha, at the time the invention was made, this was unknown. Examiner reiterates that the Application, as originally filed specifically teaches that ECPKA has been surprisingly and unexpectedly discovered (p. 3) and further teaches that antibody that distinguishes ECPKA from intracellular PKA and ectoPKA can be generated (p. 20). Given this teaching, at the time the invention was made, the identity of ECPKA and PKA catalytic subunit C alpha was unknown and therefore, at the time the invention was made, the specification as originally filed did not provide sufficient information to meet the written description requirements of 35 USC 112, first paragraph. This is especially true in view of the teaching that distinguishing antibody could be generated.

Applicant reiterates arguments stating that the claims are not directed to a novel protein. The arguments have been considered but have not been found persuasive for the reasons of record.

Applicant mischaracterizes the statements in the specification suggesting that the antibody referred to might be drawn to the regulatory subunit, rather than the catalytic subunit. It is suggested that Applicant review the specification at page 20, lines 26-28 wherein the specification proposes an antibody to the N terminal glycine of the Calpha subunit of ECPKA.

Applicant argues that the Office has not presented sufficient evidence of the lack of written description. The argument has been considered but not found persuasive given the teachings of the specification as originally filed and the post-filing reference. It is clear that the written description in the specification is not sufficient since Applicant is describing a product that appears not to exist, that is, one that is distinguishable from the Calpha subunit of intracellular PKA.

The arguments have been considered but have not been found persuasive for the reasons of record.

2. Claims 1-8 remain rejected under 35 USC 112, second paragraph for the reasons previously set forth in the paper mailed November 1, 2005, Section 6, page 10.

Applicant argues that Examiner did not address Applicant's arguments and reiterates arguments that the claims are definite. The argument has been considered but has not been found persuasive. Applicant again mischaracterizes Examiner's statements. Examiner clearly addressed the arguments and it is clear that if the limitations within the claims are not adequately described, the recitation of those limitations in the claims is indefinite. The arguments have been considered but have not been found persuasive and the rejection is maintained.

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60/551,776 11 March 2004 (11.03.2004) US
- (71) Applicant (for all designated States except US): THE UNITED STATES OF AMERICA as represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [—/US]; Office of Technology Transfer, NIH, 6011 Executive Boulevard, Suite 325, Rockville, MD 20852 (US).
- (72) Inventor; and
- (73) Inventor/Applicant (for US only): CHO-CHUNG, Yoon, S. [US/US]; 7017 Kenhill Road, Bethesda, MD 20817 (US).
- (74) Agent: JOYCE, Catherine, M.; Liniak, Berenato & White, LLC, 6550 Rock Spring Drive, Suite 240, Bethesda, MD 20817 (US).
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(54) Title: AUTOANTIBODY DETECTION FOR CANCER DIAGNOSTICS

(57) Abstract: The present invention relates to compositions and methods for the detection of anti-ECPKA autoantibodies in a biological sample, and to the use of such compositions and methods in the diagnosis of cancer in humans and non-human mammals.

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**Title of the Invention:****Autoantibody Detection for Cancer  
Diagnostics****Field of the Invention:**

- 5           The present invention relates to compositions and methods for the detection of anti-ECPKA autoantibodies in a biological sample, and to the use of such compositions and methods in the diagnosis of cancer in humans and non-human mammals.

**Cross-Reference to Related Applications**

- 10           This application claims a right of priority to United States Patent Application Serial Nos. 60/550,348, filed March 8, 2004, and 60/551,776 filed March 11, 2004, which applications are hereby incorporated by reference in their entirety.

**Statement of Governmental Interest**

- 15           This invention was funded by NCI Intramural Research Program CCR at the National Institutes of Health. The United States Government has certain rights to this invention.

**Background of the Invention:**

- 20           Tumor markers are synthesized by malignant cells and released into the bloodstream. Such markers may also be produced by host tissues in response to invasion or as a result of tumor-induced metabolic changes. Tumor marker levels in blood or tissue fluids are helpful in diagnosing, screening, and monitoring tumor progression or regression. An ideal tumor marker would allow a simple blood test to detect cancer, and its levels would correlate with the stage of tumor progression.
- 25           Due to the lack of sensitivity and specificity, however, no single marker has been previously established in a general healthy population or in most high-risk populations. The use of tumor markers in cancer diagnostics is well described

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- (Sluss, P.M. *et al.* [2004] "ESTABLISHMENT OF A CENTRAL LABORATORY SERUM TUMOR MARKER SERVICE ON A CONSOLIDATED IMMUNODIAGNOSTIC PLATFORM: DEVELOPMENT OF PRACTICE STANDARDS, SERVICE IMPROVEMENTS, AND OPERATIONAL EFFICIENCY," Clin Leadersh Manag Rev. 18[1]:25-31; Gion, M. [2000] "SERUM TUMOUR MARKERS: FROM QUALITY CONTROL TO TOTAL QUALITY MANAGEMENT," Breast 9[6]:306-11; Wiesner, A. [2004] "DETECTION OF TUMOR MARKERS WITH PROTEINCHIP<sup>®</sup> TECHNOLOGY," Curr Pharm Biotechnol. Feb;5[1]:45-67; Crawford, N.P. *et al.* [2003] "TUMOR MARKERS AND COLORECTAL CANCER: UTILITY IN MANAGEMENT," J Surg Oncol. 84[4]:239-48; Agnantis, N.J. *et al.* [2003] "TUMOR MARKERS. AN UPDATE APPROACH FOR THEIR PROGNOSTIC SIGNIFICANCE. PART I. IN VIVO," 17[6]:609-18; Riley, R.D. *et al.* [2004] "A SYSTEMATIC REVIEW OF MOLECULAR AND BIOLOGICAL TUMOR MARKERS IN NEUROBLASTOMA," Clin Cancer Res. 10[1 Pt 1]:4-12; Given, M. *et al.* [2000] "THE PREDICTIVE OF TUMOUR MARKERS CA 15-3, TPS AND CEA IN BREAST CANCER RECURRENCE," Breast. 9[5]:277-80).

- Currently available cancer markers measure cancer antigens. For example, prostate cancer can be diagnosed by measuring the prostate-specific antigen (PSA) cancer marker (Gretzer, M.B. *et al.* [2003] "PSA MARKERS IN PROSTATE CANCER DETECTION," Urol Clin North Am. 30[4]:677-86). The Carcino-Embryonic Antigen (CEA) marker has been found to have diagnostic utility in assessing colorectal cancer (Crawford, N.P. *et al.* [2003] "TUMOR MARKERS AND COLORECTAL CANCER: UTILITY IN MANAGEMENT," J Surg Oncol. 84[4]:239-48). The cancer antigen, CA15-3, has been correlated with breast cancer (Cheung, K.L. *et al.* [2003] "OBJECTIVE MEASUREMENT OF REMISSION AND PROGRESSION IN METASTATIC BREAST CANCER BY THE USE OF SERUM TUMOUR MARKERS," Minerva Chir. Jun;58[3]:297-303). The cancer antigen, CA19-9, has been employed to diagnose gastrointestinal cancer (Grotowski, M. [2002] "ANTIGENS [CEA AND CA 19-9] IN DIAGNOSIS AND PROGNOSIS COLORECTAL CANCER," Pol Merkuriusz Lek. 12[67]:77-80; Trompetas, V. *et al.* [2002] "GIANT BENIGN TRUE CYST OF THE SPLEEN WITH HIGH SERUM LEVEL OF CA 19-9," Eur J Gastroenterol Hepatol. 14[1]:85-8). The cancer antigen, CA125, has been used to diagnose

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ovarian cancer (Anderiesz, C. *et al.* [2003] "SCREENING FOR OVARIAN CANCER," Med J Aust. 178[12]:655-6).

The majority of solid tumors show chromosomal instability caused by aberrations in chromosomal segregation during cell division. Several enzymatic  
5 kinases are involved in maintaining proper chromosomal segregation and regulating cell cycle progression. One such kinase, cAMP-dependent protein kinase (PKA), appears to have a functional role in many aspects of cell signaling, metabolism, and proliferation (Matyakhina, L. *et al.* [2002] "PROTEIN KINASE A AND CHROMOSOMAL STABILITY," Ann NY Acad Sci. 968:148-57; Tortora, G. *et al.*  
10 [2002] "PROTEIN KINASE A AS TARGET FOR NOVEL INTEGRATED STRATEGIES OF CANCER THERAPY," Ann NY Acad Sci. 968:139-47).

Mammalian cells possess two types of cAMP-dependent protein kinase (PKA) species (Krebs, E.G. *et al.* [1979] "PHOSPHORYLATION-DEPHOSPHORYLATION OF ENZYMES," Annu Rev Biochem. 48:923-39). These  
15 protein kinases are designated type I (PKA-I) and type II (PKA-II); they are distinguished by different regulatory subunits (R subunits) RI and RII, and share a common catalytic subunit (C subunit) (Beebe, S.J. *et al.* [1986] "CYCLIC NUCLEOTIDE-DEPENDENT PROTEIN KINASES," In: The Enzymes: Control by Phosphorylation, E.G. Krebs *et al.* [Eds] Academic Press: Orlando and London.  
20 pp. 43-111).

Traditionally, the enzyme activity of protein kinases has been assayed by following the transfer of a radioactive phosphate group from ( $\gamma$ -<sup>32</sup>P) ATP to a residue of a suitable protein or peptide substrate (See, e.g., Witt, J.J. *et al.* [1975] Anal Biochem. 66:253-8; Casnellie, J.E. [1991] Methods Enzymol. 200:115-20;  
25 U.S. Patent No. 6,498,005). PKA enzyme assays have been described (Cohen, C.B. *et al.* [1999] "A MICROCHIP-BASED ENZYME ASSAY FOR PROTEIN KINASE A," Anal Chem. [1999] 273:89-97; Cho, Y.S. *et al.* [2000] "EXTRACELLULAR PROTEIN KINASE A AS A CANCER BIOMARKER: ITS EXPRESSION BY TUMOR CELLS AND REVERSAL BY A MYRISTATE-LACKING C<sub>ALPHA</sub> AND RII<sub>BETA</sub> SUBUNIT  
30 OVEREXPRESSION," Proc Natl Acad Sci USA. 97[2]:835-40).

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Through biochemical studies and gene cloning, four isoforms of the R subunits, RI $\alpha$ , RI $\beta$ , RII $\alpha$ , and RII $\beta$ , have been identified (Amieux, P.S. *et al* [2002] "THE ESSENTIAL ROLE OF RI ALPHA IN THE MAINTENANCE OF REGULATED PKA ACTIVITY," Ann NY Acad Sci. 968:75-95; McKnight, G.S. *et al*. [1988] 5 "ANALYSIS OF CAMP-DEPENDENT PROTEIN KINASE SYSTEM USING MOLECULAR GENETIC APPROACHES," Recent Prog Horm Res. 44:307-35; Levy, F.O. *et al*. [1988] "MOLECULAR CLONING, COMPLEMENTARY DEOXYRIBONUCLEIC ACID STRUCTURE AND PREDICTED FULL-LENGTH AMINO ACID SEQUENCE OF THE HORMONE-INDUCIBLE REGULATORY SUBUNIT OF 3',5'-CYCLIC ADENOSINE 10 MONOPHOSPHATE-DEPENDENT PROTEIN KINASE FROM HUMAN TESTIS," Mol Endocrinol. 2:1364-73).

Importantly, the ratios of PKA-I to PKA-II can change dramatically during cell development, differentiation, and transformation (Lohmann, S.M. *et al* [1984] "REGULATION OF THE CELLULAR AND SUBCELLULAR CONCENTRATIONS AND 15 DISTRIBUTION OF CYCLIC NUCLEOTIDE-DEPENDENT PROTEIN KINASES," In: Advances in Cyclic Nucleotide and Protein Phosphorylation Research, P. Greengard *et al*. [Eds] Raven Press: New York. pp. 63-117; Cho-Chung, Y.S. [1990] "ROLE OF CYCLIC AMP RECEPTOR PROTEINS IN GROWTH, DIFFERENTIATION, AND SUPPRESSION OF MALIGNANCY: NEW APPROACHES TO 20 THERAPY," Cancer Res. 50:7093-100; Cho-Chung, Y.S. [2003] "cAMP SIGNALING IN CANCER GENESIS AND TREATMENT," Cancer Treat Res. 115:123-43).

The cAMP signaling pathway has been proposed as a therapeutic target in lymphoid malignancies (Lerner, A. *et al*. [2000] "THE CAMP SIGNALING PATHWAY AS A THERAPEUTIC TARGET IN LYMPHOID MALIGNANCIES," Leuk 25 Lymphoma. 37[1-2]:39-51; Cho-Chung, Y.S. *et al*. [1995] "CAMP-DEPENDENT PROTEIN KINASE: ROLE IN NORMAL AND MALIGNANT GROWTH," Crit Rev Oncol Hematol. 21[1-3]:33-61; Cho-Chung, Y.S. *et al*. [1993] "THE REGULATORY SUBUNIT OF CAMP-DEPENDENT PROTEIN KINASE AS A TARGET FOR CHEMOTHERAPY OF CANCER AND OTHER CELLULAR DYSFUNCTIONAL-RELATED 30 DISEASES," Pharmacol Ther. 60[2]:265-88). Increased expression of RI $\alpha$ /PKA-I has been shown in human cancer cell lines and primary tumors, as compared with

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- normal counterparts (Cho-Chung, Y.S. [1990] "ROLE OF CYCLIC AMP RECEPTOR PROTEINS IN GROWTH, DIFFERENTIATION, AND SUPPRESSION OF MALIGNANCY: NEW APPROACHES TO THERAPY," *Cancer Res.* 50:7093-100; Miller, W.R. *et al.* [1993] "TYPES OF CYCLIC AMP BINDING PROTEINS IN HUMAN BREAST CANCERS,"
- 5 Eur J Cancer. 29A:989-91) in cells after transformation with chemical or viral carcinogens and the Ki-*ras* oncogene or transforming growth factor- $\alpha$ , and on stimulation of cell growth with the granulocyte-macrophage colony-stimulating factor or phorbol esters (Cho-Chung, Y.S. [1990] "ROLE OF CYCLIC AMP RECEPTOR PROTEINS IN GROWTH, DIFFERENTIATION, AND SUPPRESSION OF
- 10 MALIGNANCY: NEW APPROACHES TO THERAPY," *Cancer Res.* 50:7093-100; Cho-Chung, Y.S. *et al.* [2002] "DISSECTING THE CIRCUITRY OF PROTEIN KINASE A AND CAMP SIGNALING IN CANCER GENESIS: ANTISENSE, MICROARRAY, GENE OVEREXPRESSION, AND TRANSCRIPTION FACTOR DECOY," *Ann NY Acad Sci.* 968:22-36). Conversely, a decrease in the expression of RI $\alpha$ /PKA-I correlates with
- 15 growth inhibition induced by site-selective cAMP analogs and antisense oligonucleotides targeted against the RI $\alpha$  subunit of PKA in a broad spectrum of human cancer cell lines and human tumors grown in nude mice (Cho-Chung, Y.S. *et al.* [1989] "SITE-SELECTIVE CYCLIC AMP ANALOGS AS NEW BIOLOGICAL TOOLS IN GROWTH CONTROL, DIFFERENTIATION AND PROTO-ONCOGENE REGULATION,"
- 20 *Cancer Inv.* 7:161-77; Cho-Chung, Y.S. *et al.* [1999] "ANTISENSE DNA-TARGETING PROTEIN KINASE A-RI $\alpha$  SUBUNIT: A NOVEL APPROACH TO CANCER TREATMENT," *Front Biosci.* 4:D898-D907).

- It has been previously demonstrated that various cancer cell types excrete PKA into the conditioned medium (Cho, Y.S. *et al.* [2000] "EXTRACELLULAR
- 25 PROTEIN KINASE A AS A CANCER BIOMARKER: ITS EXPRESSION BY TUMOR CELLS AND REVERSAL BY A MYRISTATE-LACKING C $\alpha$  AND RI $\beta$  SUBUNIT OVEREXPRESSION," *Proc Natl Acad Sci USA.* 97[2]:835-40). This extracellular protein kinase A (ECPKA) is present in active, free catalytic subunit (C subunit) form ("PKA C $\alpha$ ") and its activity is specifically inhibited by the PKA inhibitory
- 30 protein PKI. Overexpression of the C $\alpha$  or RI $\alpha$  subunit gene of PKA in an expression vector, which upregulates intracellular PKA-I, markedly upregulates

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ECPKA expression. In contrast, overexpression of the RII $\beta$  subunit — which eliminates PKA-I, upregulates PKA-II, and reverts the transformed phenotype — downregulates ECPKA. A mutation in the Ca gene that prevents myristylation allows intracellular PKA upregulation but blocks the ECPKA increase, suggesting

5 that the NH<sub>2</sub>-terminal myristyl group of Ca is required for ECPKA expression. In the serum of cancer patients, ECPKA expression is markedly upregulated, in contrast to normal serum (Cho, Y.S. *et al.* [2000] "EXTRACELLULAR PROTEIN KINASE A AS A CANCER BIOMARKER: ITS EXPRESSION BY TUMOR CELLS AND REVERSAL BY A MYRISTATE-LACKING C<sub>ALPHA</sub> AND RII<sub>BETA</sub> SUBUNIT

10 OVEREXPRESSION," *Proc Natl Acad Sci USA*. 97[2]:835-40).

The development of monoclonal antibodies has led to the identification of numerous tumor-associated antigens in the serum and tissues of patients with malignancies. Protein products of oncogenes and tumor suppressor genes can be detected in extracellular fluids and serve as potential markers for carcinogenesis *in*

15 *vivo*. Some of these growth factors are encoded by oncogenes. For example, higher levels of p21-ras protein are encoded by the ras oncogenes found in patients' blood. Circulating antibodies against p53 tumor suppressor protein have been found in sera of patients with breast and lung carcinomas and in children with B-lymphomas (Winter, S.F. *et al.* [1992] "DEVELOPMENT OF ANTIBODIES AGAINST

20 P53 IN LUNG CANCER PATIENTS APPEARS TO BE DEPENDENT ON THE TYPE OF P53 MUTATION." *Cancer Res.* 52:4168-74; Lubin, R., *et al.* [1993] "ANALYSIS OF P53 ANTIBODIES IN PATIENTS WITH VARIOUS CANCERS DEFINE B-CELL EPITOPES OF HUMAN P53: DISTRIBUTION ON PRIMARY STRUCTURE AND EXPOSURE ON PROTEIN SURFACE." *Cancer Res.* 53:5872-6; Crawford, L.V., *et al.* [1982] "DETECTION OF

25 ANTIBODIES AGAINST THE CELLULAR PROTEIN P53 IN SERA FROM PATIENTS WITH BREAST CANCER." *Int. J. Cancer.* 30:403-8). Antibodies against oncogenes such as c-myc and c-myb have also been found in sera of patients with colorectal and breast tumors (Sorokine, I., K. *et al.* [1991] "PRESENCE OF CIRCULATING ANTI-C-MYB ONCOGENE PRODUCT ANTIBODIES IN HUMAN SERA." *Int. J. Cancer.* 47:665-9;

30 Ben-Mahrez, K., *et al.* [1988] "DETECTION OF CIRCULATING ANTIBODIES AGAINST C-MYC PROTEIN IN CANCER PATIENT SERA." *Br J Cancer.* 57:529-34; Ben-



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Mahrez, K., *et al.* [1990] "CIRCULATING ANTIBODIES AGAINST C-MYC ONCOGENE PRODUCT IN SERA OF COLORECTAL CANCER PATIENTS." *Int J Cancer*. 46:35-8).

In addition to the above-mentioned novel markers, some other proteins, hormones, and enzymes have been used as markers for the past 30 years. Notable among these are carcinoembryonic antigen (CEA),  $\alpha$ -fetoprotein (AFP), human chorionic gonadotropin (HCG), and prostatic acid phosphatase (PAP). Most of these markers lack specificity, however. These levels are also increased under benign conditions and during gestation. All of these markers are based on the antigen determination method; the markers are lack of specificity and sensitivity.

There is great need to discover novel biomarkers and translate them to routine clinical use. The present invention is directed to such need.

#### Summary of the Invention:

The present invention relates to a highly sensitive enzyme immunoassay (EIA) for measuring IgG antibody against extracellular protein kinase A (ECPKA). Sera from 295 patients with various types of cancer and 100 persons without cancer was tested. It was found that the frequency of anti-ECPKA IgG antibody was significantly higher in cancer patients (92%) than in those without (14%) cancer. There was no significant correlation between anti-ECPKA IgG antibody measured by EIA and ECPKA antigen measured by PKA enzymatic assay, and the anti-ECPKA IgG antibody-EIA method gave greater sensitivity and specificity than the ECPKA enzymatic assay. These results demonstrate that the approach of autoantibody analysis rather than conventional antigen analysis provides a useful approach for diagnosing cancer.

In detail, the invention provides an immunoassay that measures the presence or concentration of an anti-ECPKA autoantibody in a biological sample of a mammal, wherein the immunoassay comprises the steps of:

- (a) contacting the biological sample with an antigen specific for an anti-ECPKA autoantibody, the contacting being under conditions sufficient to permit anti-ECPKA autoantibody if present in the sample to bind to the antigen and form an antigen-anti-ECPKA autoantibody complex;

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- (b) contacting the formed antigen-anti-ECPKA autoantibody complex with an anti-ECPKA autoantibody binding molecule, the contacting being under conditions sufficient to permit the anti-ECPKA autoantibody binding molecule to bind to anti-ECPKA autoantibody of the formed antigen-anti-ECPKA autoantibody complex and form an extended complex; and
- 5 (c) determining the presence or concentration of the anti-ECPKA autoantibody in the biological sample by determining the presence or concentration of the formed extended complex.

The invention further concerns the embodiment of the above-described

10 immunoassay wherein the antigen specific for an anti-ECPKA autoantibody is an extracellular PKA protein.

The invention further concerns the embodiments of the above-described immunoassays wherein in the anti-ECPKA autoantibody is an antibody of a mammalian species that differs from that of the mammal and is specific for

15 antibodies produced by the mammal.

The invention further concerns the embodiments of the above-described immunoassays wherein the mammal is a human and the anti-ECPKA autoantibody is a human IgG antibody. The invention further concerns the embodiments of the above-described immunoassays wherein the anti-ECPKA autoantibody binding

20 molecule is detectably labeled (especially with a chemical or enzyme label).

The invention further concerns the embodiments of the above-described immunoassays wherein in the step (a), the antigen specific for an anti-ECPKA autoantibody is immobilized to a solid support prior to the contacting with the biological sample.

25 The invention further concerns the embodiments of the above-described immunoassays wherein in the step (a), the antigen specific for an anti-ECPKA autoantibody is immobilized to a solid support subsequent to the contacting with the biological sample.

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The invention further concerns the embodiments of the above-described immunoassays wherein the immunoassay is an immunochromatographic immunoassay, wherein:

- in the step (a), the biological sample is placed in contact with a first porous carrier,  
5 the first porous carrier containing a non-immobilized, labeled antigen specific for an anti-ECPKA autoantibody;  
in the step (b), the formed antigen-anti-ECPKA autoantibody complex is placed in contact with a second porous carrier, the second porous carrier being in communication with the first porous carrier, and containing an immobilized anti-  
10 ECPKA autoantibody binding molecule; and  
in the step (c), the presence or concentration of the anti-ECPKA autoantibody in the biological sample is determined by detecting the presence of the labeled antigen specific for an anti-ECPKA autoantibody in the second porous carrier.

- The invention an immunological complex comprising an antigen specific  
15 for an anti-ECPKA autoantibody bound to an anti-ECPKA autoantibody, wherein the anti-ECPKA autoantibody is additionally bound to an anti-ECPKA autoantibody binding molecule.

- The invention further concerns the embodiment of the above-described immunological complex wherein the antigen specific for an anti-ECPKA  
20 autoantibody is an extracellular PKA protein.

The invention further concerns the embodiments of the above-described immunological complexes wherein the anti-ECPKA autoantibody binding molecule is detectably labeled (especially with a chemical or enzyme label).

- The invention further concerns the embodiments of the above-described  
25 immunological complexes wherein the anti-ECPKA autoantibody binding molecule is an immunological molecule. The invention further concerns the embodiments of the above-described immunological complexes wherein the anti-ECPKA autoantibody is a human autoantibody, and the anti-ECPKA autoantibody binding molecule is an anti-human IgG antibody.

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- The invention further concerns a kit for measuring the presence or concentration of an anti-ECPKA autoantibody in a biological sample of a mammal, wherein the kit comprises a hollow casing comprising a multilayer filter system, and first and second porous carriers, wherein the second porous carrier is in communication with the first porous carrier, and the first porous carrier is in communication with the multilayer filter system, a portion of which is accessible from the casing; wherein:
- the first porous carrier contains a non-immobilized, labeled PKA  $\alpha$  or  $\alpha$  fragments; and
- the second porous carrier contains an immobilized, unlabeled antibody that binds to human IgG.

The invention further concerns the embodiment of the above-described kit wherein the labeled PKA  $\alpha$  is ECPKA (especially a chemical or enzyme label).

- The invention further concerns the embodiments of the above-described kits wherein the kit detects human anti-ECPKA autoantibodies, and the antibody that binds to human IgG is an antibody of a nonhuman mammal.

#### Brief Description of the Figures:

- Figure 1 shows values obtained using a preferred immunoassay format of the present invention with normal individuals and cancer patients. Both frequency and mean titer of the patients are significantly higher (frequency = 92%, mean titer 2.2) than those of the normal controls (frequency 14%, mean titer 0.6). Values greater than 1.0 (above the dashed line) are positive.

- Figure 2 shows a receiver operating characteristic (ROC) curve of an anti-ECPKA autoantibody ELISA (Figure 1). At the point of intersection, the cutoff value for the ELISA assay is 1.0 titer, and the sensitivity and specificity are 90% and 89%, respectively.

Figure 3 shows the results of a Western blotting analysis of anti-ECPKA autoantibody in cancer patients' serum. The lanes containing M (mw marker) and  $\alpha$  (1  $\mu$ g) are stained with Coomassie Blue; strip 1 was blotted with Santa Cruz Ab

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anti-Ca antibody (Santa Cruz Biotechnology, Santa Cruz, CA); strips 2-7 were blotted with cancer patients' serum (10,000-fold dilution); strips 8-12 were blotted with normal person serum (10,000-fold dilution); strips 1-12 contained purified PKA Ca (1 µg each).

5           Figure 4 shows values obtained using an ECPKA enzymatic assay as a control of the sensitivity and discriminatory ability of the immunoassay formats of the present invention. Values obtained from normal individuals and cancer patients are shown (cancer patients, frequency = 83%, mean value = 130 mU/ml; normal individual controls, frequency = 21%, mean value = 60 mU/ml) indicating a lack  
10 of sensitivity and specificity. Values greater than 75 mU/ml (above the dashed line) are positive.

          Figure 5 shows the results of a correlation study between anti-ECPKA antibody titer and ECPKA enzymatic activity in sera from cancer patients and healthy persons. Coefficients of 0.003 for cancer patients and 0.001 for healthy  
15 persons are statistically insignificant.

          Figure 6 shows the titer of anticancer antigen antibodies in sera from cancer patients and healthy persons using HCT-15 tumor extracts as a source of cancer antigens. Values >1.5 (broken line) are positive.

          Figure 7 shows the observed titers of antigen antibodies in sera from  
20 cancer patients with different types of cancers (lung, renal, pancreatic, ovarian, colon, liver, gastric, bladder, and cervical carcinomas, melanoma, sarcoma, and leiomyoma).

### **Description of the Preferred Embodiments:**

          The present invention relates to compositions and methods for the detection  
25 of anti-ECPKA autoantibodies in a biological sample, and to the use of such compositions and methods in the diagnosis of cancer in humans and non-human mammals (especially canine, feline, bovines, ovine, porcine, and equine mammals).

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An extracellular form of PKA (ECPKA) is secreted from cancer cells (Cho, Y.S. *et al.* [2000] "EXTRACELLULAR PROTEIN KINASE A AS A CANCER BIOMARKER: ITS EXPRESSION BY TUMOR CELLS AND REVERSAL BY A MYRISTATE-LACKING C<sub>ALPHA</sub> AND RII<sub>BETA</sub> SUBUNIT OVEREXPRESSION," Proc Natl Acad Sci USA. 97[2]:835-40). The present invention derives in part from the recognition that ECPKA secretion elicits the formation of serum autoantibodies and that the presence and/or concentration of such autoantibodies can serve as a cancer diagnostic and prognostic marker. As described herein, the invention provides a highly sensitive immunoassay that measures the concentration and/or presence of anti-ECPKA autoantibody (especially an anti-ECPKA IgG antibody) in biological samples of suspected and confirmed cancer patients. This autoantibody-based immunoassay method provides a routine diagnostic procedure for detecting various cancer cell types.

The present invention concerns the binding of antigens and antibodies. As used herein, an "epitope" is a 2- or 3-dimensional region of an antigen that is recognized by and that specifically binds to an antibody. As used herein, an antigen and antibody are said to be "specific" for one another, or to "recognize" one another, or to "bind" to one another if they are capable of immunospecific binding to one another.

Any of a wide variety of assay formats may be used in accordance with the methods of the present invention. Such formats may be heterogeneous or homogeneous, sequential or simultaneous, competitive or noncompetitive. U.S. Patent Nos. 5,563,036; 5,627,080; 5,633,141; 5,679,525; 5,691,147; 5,698,411; 5,747,352; 5,811,526; 5,851,778; and 5,976,822 illustrate several different assay formats and applications. Such assays can be formatted to be quantitative, to measure the concentration or amount of an anti-ECPKA autoantibody, or they may be formatted to be qualitative, to measure the presence or absence of an anti-ECPKA autoantibody.

Heterogeneous immunoassay techniques typically involve the use of a solid phase material to which the reaction product becomes bound, but may be adapted

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to involve the binding of nonimmobilized antigens and antibodies (i.e., a solution-phase immunoassay). The reaction product is separated from excess sample, assay reagents, and other substances by removing the solid phase from the reaction mixture (e.g., by washing). One type of solid phase immunoassay that may be used  
5 in accordance with the present invention is a sandwich immunoassay. In the sandwich assay, the more analyte present in the sample, the greater the amount of label present on the solid phase. This type of assay format is generally preferred, especially for the visualization of low analyte concentrations, because the appearance of label on the solid phase is more readily detected.

10 In accordance with a preferred embodiment of the present invention, antigen that is specifically reactive with an anti-ECPKA autoantibody is bound to a solid support (i.e., immobilized) and incubated in contact with the biological sample being tested for the presence of anti-ECPKA IgG antibody. As will be appreciated, the antigen may be incubated with the biological sample in an  
15 unbound state and then subsequently bound to the solid support (i.e., immobilizable). The supports are then preferably extensively treated (e.g., by washing, etc.) to substantially remove non-ECPKA IgG antibodies that may be present but which failed to bind to the bound antigen. In consequence of such treatment, an immune complex forms between the antigen and anti-ECPKA IgG  
20 antibody.

A detectably labeled second antibody (e.g., an anti-human IgG antibody) is then preferably added and the support is incubated under conditions sufficient to permit the second antibody to bind to any anti-ECPKA IgG antibody that may be present. The support is then preferably extensively treated (e.g., by washing, etc.)  
25 to substantially remove any unbound second antibody. If the anti-ECPKA IgG antibody is present in the test sample, then the two antibodies will form an immune complex with the analyte (i.e., a second antibody/anti-ECPKA IgG antibody/antigen sandwich). In such an assay, the detection of second antibody bound to the support is indicative of anti-ECPKA IgG antibody in the fluid being  
30 tested. Sandwich assay formats are described by Schuurs *et al.* U.S. Patent Nos. 3,791,932 and 4,016,043, and by Pankratz, *et al.*, U.S. Patent No. 5,876,935. The

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second antibody may be a natural immunoglobulin isolated from nonhuman primates (e.g., anti-human IgG murine antibody, anti-human IgG goat antibody, etc.), or can be produced recombinantly or synthetically. It may be an intact immunoglobulin, or an immunoglobulin fragment (e.g., Fab, F[Ab]<sub>2</sub>, etc.). As  
5 desired, other binding molecules (capable of binding to anti-ECPKA autoantibodies) may be employed in concert with or in lieu of such second antibodies. For example, the anti-ECPKA autoantibodies can be biotinylated and the second antibody can be replaced with labeled avidin or streptavidin.

To eliminate the bound-free separation step and reduce the time and  
10 equipment needed for a chemical binding assay, a homogeneous assay format may alternatively be employed. In such assays, one component of the binding pair may still be immobilized; however, the presence of the second component of the binding pair is detected without a bound-free separation. Examples of  
homogeneous optical methods are the EMIT method of Syva, Inc. (Sunnyvale,  
15 CA), which operates through detection of fluorescence quenching; the laser nephelometry latex particle agglutination method of Behringwerke (Marburg, Germany), which operates by detecting changes in light scatter; the LPIA latex particle agglutination method of Mitsubishi Chemical Industries (Tokyo, Japan); the TDX fluorescence depolarization method of Abbott Laboratories (Abbott Park,  
20 IL); and the fluorescence energy transfer method of Cis Bio International (Paris, France). Any of such assays may be adapted for use in accordance with the objectives of the present invention.

The binding assay of the present invention may be configured as a competitive assay. In a competitive assay, the more anti-ECPKA IgG antibody  
25 present in the test sample, the lower the amount of label present on the solid phase.

In a manner similar to the sandwich assay, the competitive assay can be conducted by providing a defined amount of a labeled anti-ECPKA IgG antibody and determining whether the fluid being tested contains anti-ECPKA IgG antibody that would compete with the labeled antibody for binding to the support. In such a  
30 competitive assay, the amount of captured labeled antibody is inversely



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proportional to the amount of analyte present in the test sample. Smith (U.S. Patent No. 4,401,764) describes an alternative competitive assay format using a mixed binding complex that can bind analyte or labeled analyte but in which the analyte and labeled analyte cannot simultaneously bind the complex. Clagett (U.S. Patent

5 No. 4,746,631) describes an immunoassay method using a reaction chamber in which an analyte/ligand/marker conjugate is displaced from the reaction surface in the presence of test sample analyte and in which the displaced

analyte/ligand/marker conjugate is immobilized at a second reaction site. The conjugate includes biotin, bovine serum albumin, and synthetic peptides as the

10 ligand component of the conjugate, and enzymes, chemiluminescent materials, enzyme inhibitors, and radionucleotides as the marker component of the conjugate. Li (U.S. Patent No. 4,661,444) describes a competitive immunoassay using a

conjugate of an anti-idiotypic antibody and a second antibody, specific for a detectable label, in which the detectable response is inversely related to the

15 presence of analyte in the sample. Allen (European Patent Appl. No. 177,191) describes a binding assay involving a conjugate of a ligand analog and a second reagent, such as fluorescein, in which the conjugate competes with the analyte (ligand) in binding to a labeled binding partner specific for the ligand, and in which

the resultant labeled conjugate is then separated from the reaction mixture by

20 means of solid phase carrying a binding partner for the second reagent. This binding assay format combines the use of a competitive binding technique and a reverse sandwich assay configuration; i.e., the binding of conjugate to the labeled binding member prior to separating conjugate from the mixture by the binding of the conjugate to the solid phase. The assay result, however, is determined as in a

25 conventional competitive assay in which the amount of label bound to the solid phase is inversely proportional to the amount of analyte in the test sample. Chieragatt *et al.* (GB Patent No. 2,084,317) describe a similar assay format using an indirectly labeled binding partner specific for the analyte. Mochida *et al.* (U.S. Patent No. 4,185,084) also describe the use of a double-antigen conjugate that

30 competes with an antigen analyte for binding to an immobilized antibody and that is then labeled. This method also results in the detection of label on a solid phase in which the amount of label is inversely proportional to the amount of analyte in the

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test sample. Sadeh *et al.* (U.S. Patent No. 4,243,749) describe a similar enzyme immunoassay in which a hapten conjugate competes with analyte for binding to an antibody immobilized on a solid phase. Any of such variant assays may be used in accordance with the present invention.

- 5 In all such assay formats, at least one component of the assay reagents will preferably be labeled or otherwise detectable by the evolution or quenching of light. Such component may be a second antibody, anti-ECPKA IgG antibody, or an antigen that binds to an anti-ECPKA IgG antibody, depending on the immunoassay format employed. Radioisotopic-binding assay formats (e.g., a radioimmunoassay, etc.) employ a radioisotope as such label; the signal is detectable by the evolution of light in the presence of a fluorescent or fluorogenic moiety (see Lucas *et al.* [U.S. Patent No. 5,698,411] and Landrum *et al.* [U.S. Patent No. 5,976,822]). Enzymatic-binding assay formats (e.g., an ELISA, etc.) employ an enzyme as a label; the signal is detectable by the evolution of color or light in the presence of a chromogenic or fluorogenic moiety. Other labels, such as paramagnetic labels, materials used as colored particles, latex particles, colloidal metals such as selenium and gold, and dye particles (see U.S. Patent Nos. 4,313,734; 4,373,932, and 5,501,985) may also be employed. The use of enzymes (especially alkaline phosphatase,  $\beta$ -galactosidase, horse radish peroxidase, or urease) as the detectable label (i.e., an enzyme immunoassay or EIA) is preferred.

- The presence of enzymatic labels may be detected through the use of chromogenic substrates (including those that evolve or adsorb fluorescent, UV, visible light, etc.) in response to catalysis by the enzyme label. More preferably, chemical labels may be employed (e.g., colloidal gold, latex bead labels, etc.).
- 25 Detection of label can be accomplished using multiple detectors, multipass filters, gratings, or spectrally distinct fluors (see e.g., U.S. Patent No. 5,759,781), etc. It is particularly preferred to employ peroxidase as an enzyme label, especially in concert with the chromogenic substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB). In the case of labeling of the antibodies with peroxidase as enzyme, it is possible to
- 30 use the periodate technique (Nakane, P.K. *et al.* [1974] "PEROXIDASE-LABELED ANTIBODY. A NEW METHOD OF CONJUGATION," J Histochem Cytochem. 22:1084-

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90) or a method reported in which the partners are linked with a heterobifunctional reagent (Ishikawa, E. *et al.* [1983] "ENZYME-LABELING OF ANTIBODIES AND THEIR FRAGMENTS FOR ENZYME IMMUNOASSAY AND IMMUNOHISTOCHEMICAL STAINING," *J Immunoassay*. 4[3]:209-327).

5 Any of a wide variety of solid supports may be employed in the immunoassays of the present invention. Suitable materials for the solid support are synthetics such as polystyrene, polyvinyl chloride, polyamide, or other synthetic polymers, natural polymers such as cellulose, as well as derivatized natural polymers such as cellulose acetate or nitrocellulose, and glass, especially glass  
10 fibers. The support can take the form of spheres, rods, tubes, and microassay or microtiter plates. Sheet-like structures such as paper strips, small plates, and membranes are likewise suitable. The surface of the carriers can be permeable and impermeable for aqueous solutions.

Although the foregoing description pertains to assaying for the presence of  
15 anti-ECPKA autoantibodies in biological samples that are fluids (e.g., sera, blood, urine, saliva, pancreatic juice, cerebrospinal fluid, semen, etc.), it will be appreciated that any fluidic biological sample (e.g., tissue or biopsy extracts, extracts of feces, sputum, etc.) may likewise be employed in the assays of the present invention. Most preferably, the biological sample being assayed will be  
20 serum.

Materials for use in the assay of the invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement; one or more containers means  
vials, tubes and the like; each of the containers means comprising one of the  
25 separate elements to be used in the method. For example, one of the containers means may comprise a suitable antigen (such as ECPKA (PKA C $\alpha$ , or C $\alpha$  fragments) or an extract of one or more different types of cancer cells and tumors) bound to a solid support. A second container may comprise soluble, detectably labeled second antibody, preferably in lyophilized form, or in solution. In addition,  
30 the kit may also contain one or more containers, each of which comprises a

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(different) predetermined amount of ECPKA (PKA Ca, or Ca fragments) or anti-ECPKA (PKA Ca) autoantibody. These latter containers can be used to prepare a standard curve into which can be interpolated the results obtained from the sample containing the unknown amount of autoantibodies to ECPKA.

- 5 In using the kit, all the user has to do is add to a container a premeasured amount of a sample suspected of containing a measurable yet unknown amount of autoantibodies to ECPKA, a premeasured amount of support-bound antigen present in the first container, and a premeasured amount of the detectably labeled second antibody present in the second container. After an appropriate time for
- 10 incubation, an immune complex is formed and is separated from the supernatant fluid, and the immune complex or the supernatant fluid are detected, as by radioactive counting, addition of an enzyme substrate, and color development, or by inclusion of a chemical label (e.g., colloidal gold, latex beads, etc.).

- The present invention particularly relates to the use of immuno-
- 15 chromatographic assay formats to detect anti-ECPKA autoantibodies. In a preferred immunochromatographic assay format, two contacting, but spatially distinct, porous carriers are employed. The first such carrier will contain a non-immobilized, labeled PKA Ca or Ca fragments (e.g., ECPKA [Ca] or protease digests or Ca) and the second such carrier will contain an immobilized, but
- 20 unlabeled antibody that binds to IgG (e.g., where human anti-ECPKA autoantibodies are being assayed, the unlabeled antibody may be an anti-human IgG antibody).

- Preferably, the device will comprise a hollow casing constructed of, for example, a plastic material, etc., in which the first carrier will communicate
- 25 indirectly with the interior of the casing via a multilayer filter system that is accessible from the device (e.g., by protruding therefrom or by being incompletely covered by the device), such that a serum, plasma, or whole blood test sample can be applied directly to the filter system and will permeate therefrom into the first porous carrier. In such a device, the permeation of fluid containing anti-ECPKA
- 30 autoantibodies will cause the non-immobilized labeled PKA Ca or Ca fragments of

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the first carrier to become bound to the migrating antibodies, and will then permeate into the second carrier. Because the second carrier contains immobilized antibody that binds human IgG, any labeled PKA C $\alpha$  or C $\beta$  fragments entering the second carrier will be entrapped therein.

5           Detection of labeled PKA C $\alpha$  or C $\beta$  fragments in the carrier containing the immobilized unlabeled antibody thus indicates that anti-ECPKA autoantibodies are present in the sample being evaluated. The assay can be made quantitative by measuring the quantity of labeled PKA C $\alpha$  or C $\beta$  fragments that become bound within the second porous carrier.

10           Having now generally described the invention, the same will be more readily understood through reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention, unless specified.

#### Example 1

15           **Comparative Evaluation of Anti-ECPKA Autoantibody Immunoassay and PKA Enzymatic Assay in Diagnosing Cancer In Humans**

##### Materials and Methods

**Subjects:** Serum samples are taken from 295 human patients with various cancer cell types and from 100 persons without cancer.

20           **Enzyme-linked immunoabsorbent assay (ELISA):** Anti-ECPKA IgG autoantibody in the sera of cancer patients and healthy individuals are measured by solid-phase ELISA assay. For such assays, round-bottom polyvinyl chloride microtiter plates (Thermolab System, Helsinki, Finland) are coated with 100  $\mu$ l of diluted (50  $\mu$ g per ml concentration with PBS) purified recombinant human PKA  
25 C $\alpha$  antigen (see Purification of PKA C $\alpha$ , Materials and Methods). The plates are incubated at room temperature for 1 hour, then washed one time with washing buffer (20 mM Hepes, 0.9% NaCl, 30 mM sucrose-0.1 % bovine serum albumin [BSA], pH 7.0), and each well is blocked for 2 hours at room temperature with 100

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µl of Blockace (Serotec, <http://www.serotec.com>) dissolved in water (4 g/400 ml), then the plates are washed two times with Na citrate washing solution (50 mM Na citrate, 0.15 M NaCl, 0.1 % polyoxyethylenesorbitan monolaurate Tween® 20, pH 5.0-5.2). The serum samples are diluted to 25,000-fold with sample dilution buffer  
5 (PBS pH 7.4, 0.25% BSA [fatty acid free fraction V], 0.05% Tween 20), and 100 µl of diluted samples are added to each well, then incubated for 1 hour at 37°C. After three washes with the Na citrate washing solution, 100 µl of 20,000-fold diluted anti-human IgG-HRP antibody-enzyme conjugate (Jackson  
ImmunoResearch Laboratories, West Grove, PA) in PBS, 1% BSA is added to the  
10 wells, after which the plates are incubated for 1 hour at room temperature. After washing the plates five times in Na citrate washing solution, 100 µl of TMB substrate is added. The reaction is stopped with 100 µl of 0.45 M H<sub>2</sub>SO<sub>4</sub> reagent, after which the absorbance at 450 nm is recorded on an ELISA reader (BioRad [Hercules, CA] microplate reader benchmark). ELISA specificity is confirmed by  
15 performing the inhibition test simultaneously on serum samples that have been incubated for 1 hr at room temperature with the above antigen at a final concentration of 1 mg/mL.

**Purification of PKA Cα:** The recombinant human PKA Cα (1.1 kb) from OT1529-Cα plasmid (Cho, Y.S. *et al.* [2000] "EXTRACELLULAR PROTEIN KINASE A  
20 AS A CANCER BIOMARKER: ITS EXPRESSION BY TUMOR CELLS AND REVERSAL BY A MYRISTATE- LACKING C<sub>ALPHA</sub> AND RII<sub>BETA</sub> SUBUNIT OVEREXPRESSION," Proc Natl Acad Sci USA. 97[2]:835-40) is infused with pQE31 DNA leading to production of pQE-Cα (Hong, S.H. Seoul National University, Seoul, Korea, unpublished). pQE-Cα plasmid was expressed in E coli and purification of native PKA Cα  
25 protein was achieved (Paragon, Baltimore MD).

**PKA Assays:** The enzyme activity of PKA is measured by the method of Rohlf, C. *et al.* (1993) ("8-Cl-CAMP INDUCES TRUNCATION AND DOWN-  
REGULATION OF THE RI ALPHA SUBUNIT AND UP-REGULATION OF THE RII BETA  
SUBUNIT OF CAMP-DEPENDENT PROTEIN KINASE LEADING TO TYPE II  
30 HOLOENZYME-DEPENDENT GROWTH INHIBITION AND DIFFERENTIATION OF HL-60 LEUKEMIA CELLS," J Biol Chem. 268:5774-82). For the measurement of serum

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ECPKA activity, the assay (total volume, 50  $\mu$ l) is carried out for 20 min at 37°C in the reaction mixture (containing 50 mM Tris HCl [pH 7.5], 1 mM DTT, 10 mM MgCl<sub>2</sub>, 5  $\mu$ M Kemptide [a synthetic substrate for PKA; GIBCO/BRL], 1.2  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP [25 Ci/mmol; ICN] with or without 5  $\mu$ M cAMP and 5  $\mu$ M PKI), and 10  $\mu$ l of serum. After incubation, the reaction mixtures are spotted onto phosphocellulose disks (GIBCO/BRL) and washed three times in 0.5% phosphoric acid. Filters are air dried and counted by liquid scintillation counter. One unit of PKA activity is defined as the amount of enzyme that transferred 1 pmol of <sup>32</sup>P from ( $\gamma$ -<sup>32</sup>P)ATP to recovered protein in 1 min at 37°C in the standard assay system. LDH activity is measured by the use of a commercial kit (Sigma).

### Results

The sera of human patients and normal control individuals is tested using the above-described ELISA, expressing anti-ECPKA autoantibody titers arbitrarily as ratios to the mean absorbance of the normal control sera. From anti-ECPKA autoantibody titers of normal controls, a ratio greater than 1.0 is considered positive.

The assay is found to be reproducible, with within-run and between-run CVs of <8.8% and <9.0%, respectively. Values for anti-ECPKA autoantibody in sera from cancer patients are shown in Figure 1. Both frequency and mean titer of the patients are significantly higher (frequency = 92%, mean titer 2.2) than those of the normal controls (frequency 14%, mean titer 0.60).

The sensitivity and specificity of the ELISA assay calculated at different cut-off values are graphically presented in a receiver operating characteristic (ROC) curve (Figure 2). At the point of interaction, the cut-off value for anti-ECPKA autoantibody titer is 1.0 (Figure 1) and the sensitivity and specificity for the ELISA test are 90% and 89%, respectively.

Immunoblotting identified anti-ECPKA autoantibody in cancer patients' serum (Figure 3). Randomly selected patients sera exhibited immunocross-reactivity toward the purified PKA C $\alpha$  protein (40 kDa) (Figure 3, strips 2-7),

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while no such immunocross-reactivity for Ca protein was observed in normal serum (Figure 3, strips 8–12).

The ECPKA enzymatic assay (which measures antigen concentration) gives a significant overlap between cancer patients (n=66) and normal controls (n=66) in frequency and mean values (patients, frequency = 83%, mean value = 130 mU/ml; normal controls, frequency = 21%, mean value = 60 mU/ml) indicating a lack of sensitivity and specificity (Figure 4).

A comparison of individual anti-ECPKA autoantibody titers obtained by ELISA and ECPKA measured by PKA enzymatic assay is shown in Figure 5.

There is no correlation between the titers of anti-ECPKA IgG antibody obtained by ELISA and ECPKA measured by enzymatic assay (ECPKA antigen measurement).

#### Example 2 Conclusions Regarding Anti-ECPKA Autoantibody Immunoassay and Its Use in Cancer In Humans

The present invention demonstrates that the sera presence of autoantibody directed against ECPKA is highly correlative of cancer. The ELISA developed for anti-ECPKA autoantibody is highly sensitive and specific, exhibiting markedly high anti-ECPKA autoantibody titers (mean titer 2.2, frequency 92%) in cancer patients, but exhibits low or negative titers and frequency (14%) in normal individual controls. A comparison of the frequency detected by ELISA with that detected by enzymatic assay shows that the ELISA that detects the autoantibody for ECPKA is highly sensitive and specific compared with the enzymatic assay that measures antigen activity.

To examine whether the autoantibody detection method of the present invention can be extended to other cancer antigens (extracellular secreted), the following experiment is performed: HCT-15 tumor (a multidrug-resistant human colon carcinoma grown in nude mice) is used as a cancer antigen source. The tumor is homogenized in Buffer #10 (20 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.5% sodium deoxycholate, 5 mM MgCl<sub>2</sub>, protease inhibitor cocktail set 1



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[Calbiochem]) (1:3), 1 part tumor (weight), 3 parts buffer (volume). The homogenate is centrifuged for 10 min at 10,000 rpm and the supernatant is collected (Extract). The Extract is incubated with protein-A sepharose (3:1), rotating 1 hr, 4°C, and the preparation is centrifuged for 2 min at 6,000 rpm. The supernatant is collected and dialyzed against PBS overnight at 4°C. After such dialysis, the extract is ready to use for coating microtiter plates in an ELISA. Figure 6 shows the titer of anticancer antigen antibodies in sera from cancer patients (n = 36) and healthy persons (n = 25). Values >1.5 (broken line) are positive. Figure 7 shows the observed titers of antigen antibodies in sera from cancer patients with different types of cancer (lung, renal, melanoma, pancreatic, ovarian, colon, liver, gastric, bladder, and cervical carcinomas, and sarcoma and leiomyoma. The patients' sera (n = 36) and normal sera (n=25) are the same as those of Figure 6.

The results demonstrate that it is possible to use the autoantibody detection method of the present invention to detect cancer antigens in humans and animals. The results indicate that ELISA for detecting autoantibodies rather than antigens for ECPKA and other cancer antigens provides a new approach for diagnosing cancer in humans and animals.

All publications and patents mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth.

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**What Is Claimed Is:**

**Claim 1.** An immunoassay that measures the presence or concentration of an anti-ECPKA autoantibody in a biological sample of a mammal, wherein said immunoassay comprises the steps of:

- 5 (a) contacting said biological sample with an antigen specific for an anti-ECPKA autoantibody, said contacting being under conditions sufficient to permit anti-ECPKA autoantibody if present in said sample to bind to said antigen and form an antigen-anti-ECPKA autoantibody complex;
- 10 (b) contacting said formed antigen-anti-ECPKA autoantibody complex with an anti-ECPKA autoantibody binding molecule, said contacting being under conditions sufficient to permit said anti-ECPKA autoantibody binding molecule to bind to anti-ECPKA autoantibody of said formed antigen-anti-ECPKA autoantibody complex and form an extended complex; and
- 15 (c) determining the presence or concentration of said anti-ECPKA autoantibody in said biological sample by determining the presence or concentration of said formed extended complex.
- 20

**Claim 2.** The immunoassay of claim 1, wherein said antigen specific for an anti-ECPKA autoantibody is an extracellular PKA protein.

**Claim 3.** The immunoassay of claim 1, wherein in said anti-ECPKA autoantibody is an antibody of a mammalian species that differs from that of said mammal and is specific for antibodies produced by said mammal.

25

**Claim 4.** The immunoassay of claim 3, wherein said mammal is a human and said anti-ECPKA autoantibody is a human IgG antibody.

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- Claim 5. The immunoassay of claim 1, wherein said anti-ECPKA autoantibody binding molecule is detectably labeled.
- Claim 6. The immunoassay of claim 5, wherein said detectable label is a chemical label.
- 5 Claim 7. The immunoassay of claim 1, wherein in said step (a), said antigen specific for an anti-ECPKA autoantibody is immobilized to a solid support prior to said contacting with said biological sample.
- Claim 8. The immunoassay of claim 1, wherein in said step (a), said antigen specific for an anti-ECPKA autoantibody is immobilized to a solid  
10 support subsequent to said contacting with said biological sample.
- Claim 9. The immunoassay of claim 1, wherein said immunoassay is an immunochromatographic immunoassay, wherein:  
in said step (a), said biological sample is placed in contact with a  
first porous carrier, said first porous carrier containing a non-  
15 immobilized, labeled antigen specific for an anti-ECPKA autoantibody;  
in said step (b), said formed antigen-anti-ECPKA autoantibody complex is placed in contact with a second porous carrier, said second porous carrier being in communication with said first  
20 porous carrier, and containing an immobilized anti-ECPKA autoantibody binding molecule; and  
in said step (c), the presence or concentration of said anti-ECPKA autoantibody in said biological sample is determined by  
25 detecting the presence of said labeled antigen specific for an anti-ECPKA autoantibody in said second porous carrier.
- Claim 10. An immunological complex comprising an antigen specific for an anti-ECPKA autoantibody bound to an anti-ECPKA autoantibody, wherein said anti-ECPKA autoantibody is additionally bound to an anti-ECPKA autoantibody binding molecule.

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- Claim 11. The immunological complex of claim 10, wherein said antigen specific for an anti-ECPKA autoantibody is an extracellular PKA protein.
- 5 Claim 12. The immunological complex of claim 10, wherein said anti-ECPKA autoantibody binding molecule is detectably labeled.
- Claim 13. The immunological complex of claim 12, wherein detectable label is a chemical label.
- Claim 14. The immunological complex of claim 10, wherein said anti-ECPKA autoantibody binding molecule is an immunological molecule.
- 10 Claim 15. The immunological complex of claim 14, wherein said anti-ECPKA autoantibody is a human autoantibody, and said anti-ECPKA autoantibody binding molecule is an anti-human IgG antibody.
- 15 Claim 16. A kit for measuring the presence or concentration of an anti-ECPKA autoantibody in a biological sample of a mammal, wherein said kit comprises a hollow casing comprising a multilayer filter system, and first and second porous carriers, wherein said second porous carrier is in communication with said first porous carrier, and said first porous carrier is in communication with said multilayer filter system, a portion of which is accessible from said casing; wherein:
- 20 said first porous carrier contains a non-immobilized, labeled PKA  $\text{C}\alpha$  or  $\text{C}\alpha$  fragments; and
- said second porous carrier contains an immobilized, unlabeled antibody that binds to human IgG.
- 25 Claim 17. The kit of claim 16, wherein said labeled PKA  $\text{C}\alpha$  is ECPKA.
- Claim 18. The kit of claim 16, wherein said label of said labeled PKA  $\text{C}\alpha$  is a chemical label.

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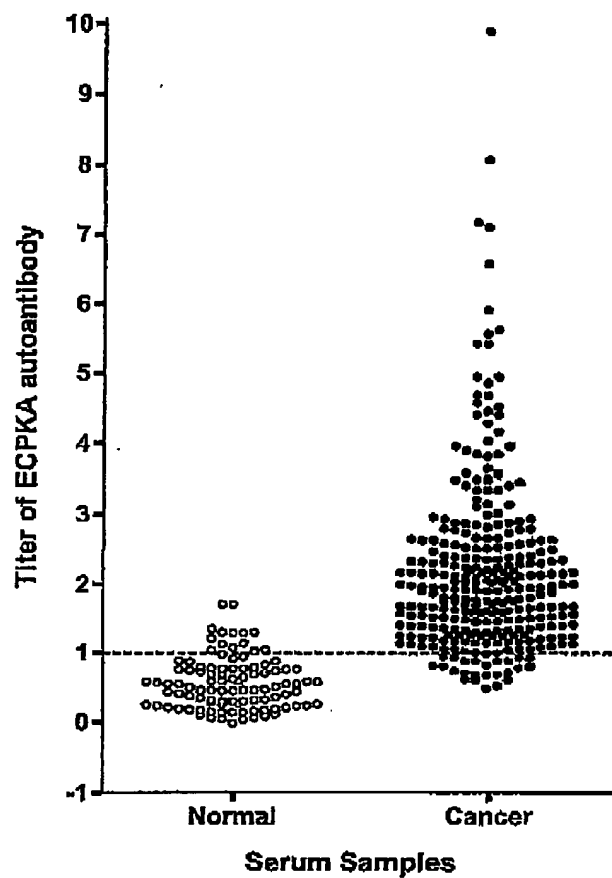
**Claim 19. The kit of claim 16, wherein said kit detects human anti-ECPKA autoantibodies, and said antibody that binds to human IgG is an antibody of a nonhuman mammal.**

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Figure 1

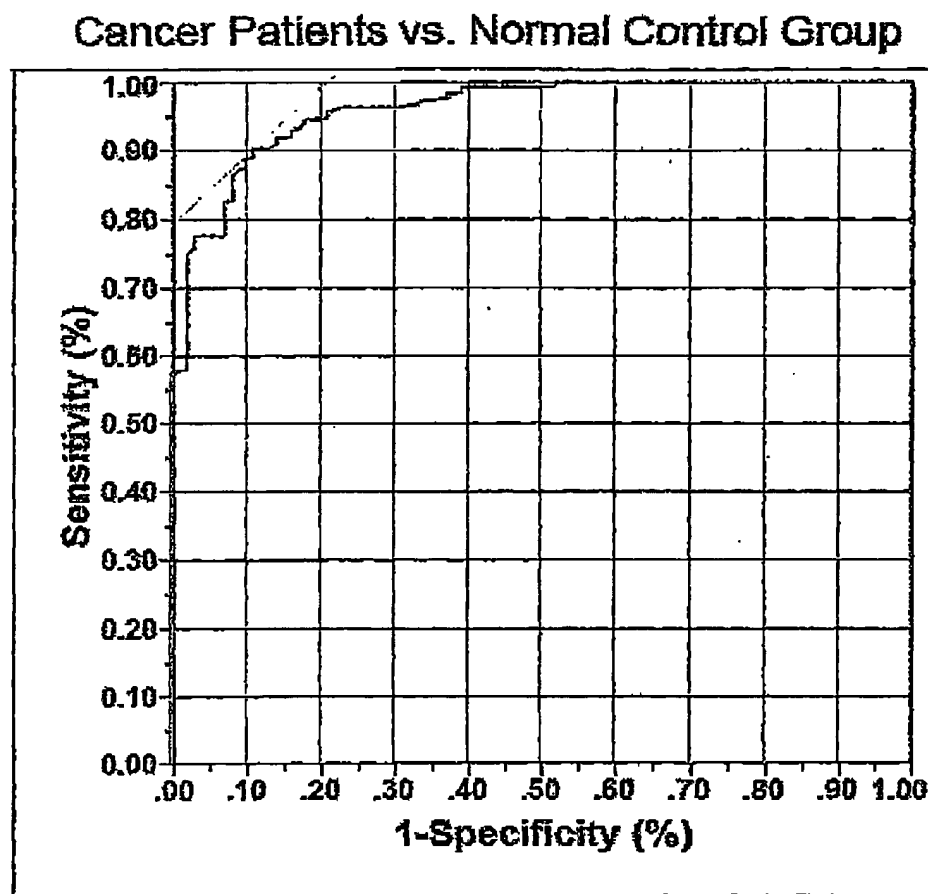


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Figure 2

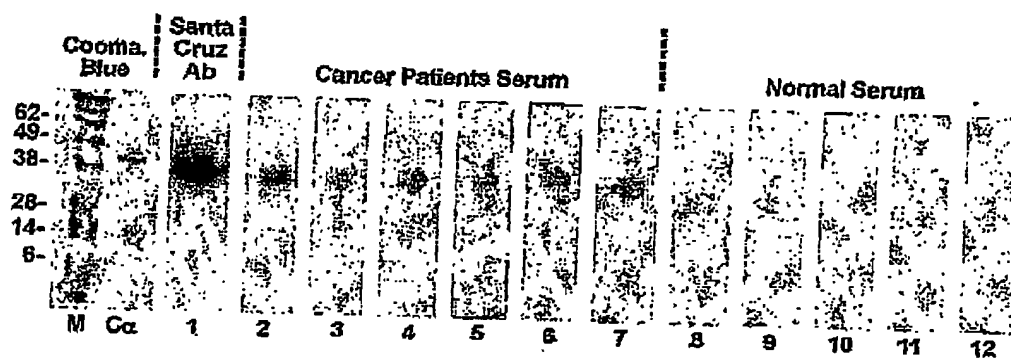


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Figure 3



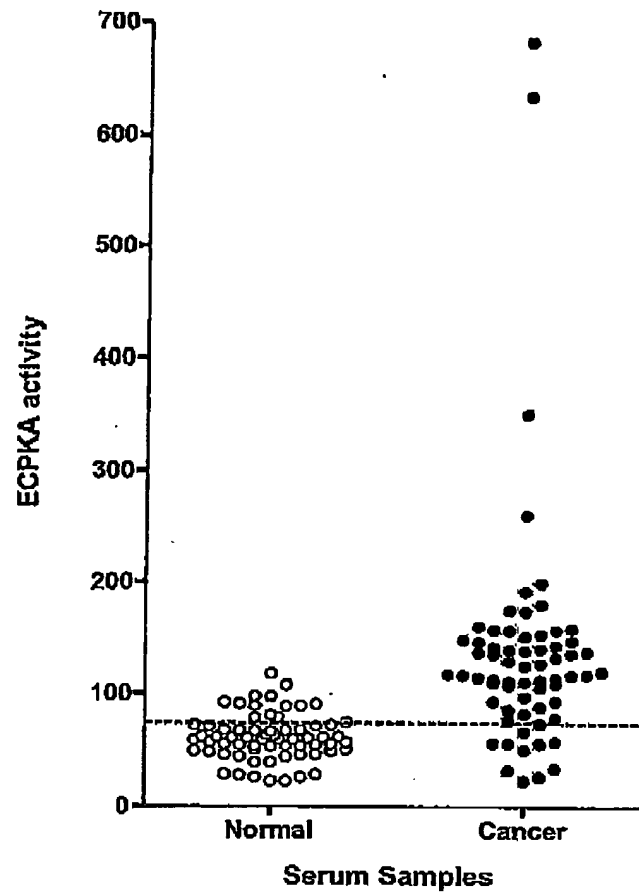


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Figure 4

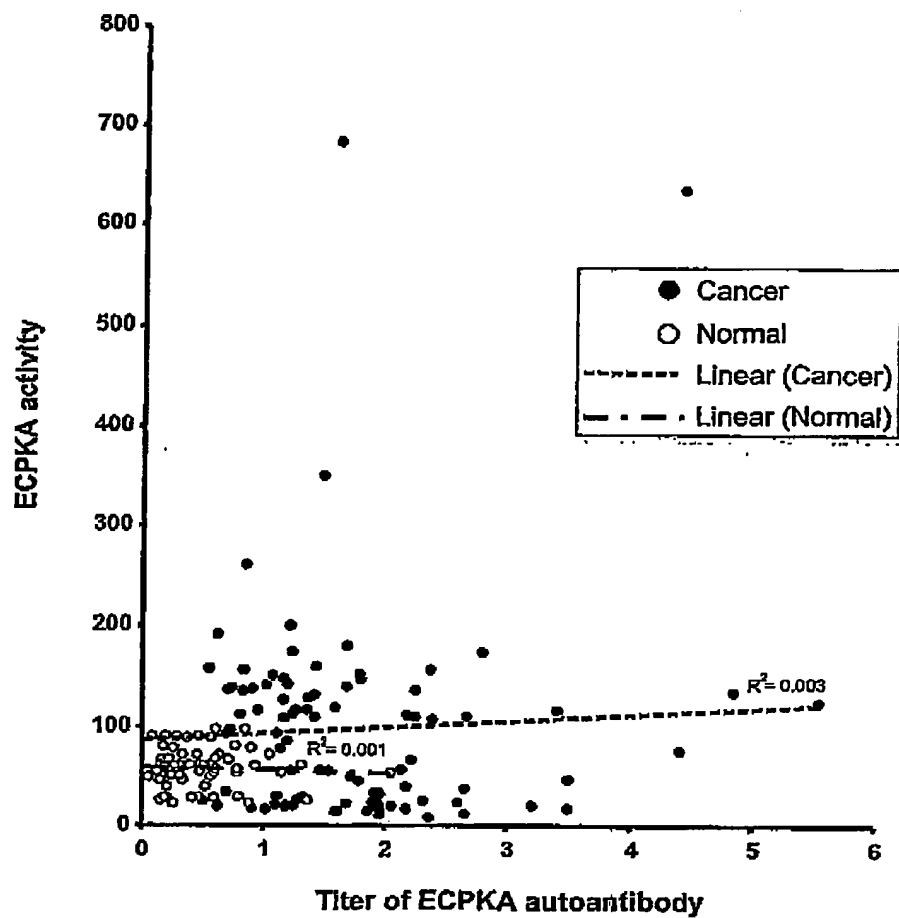


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Figure 5

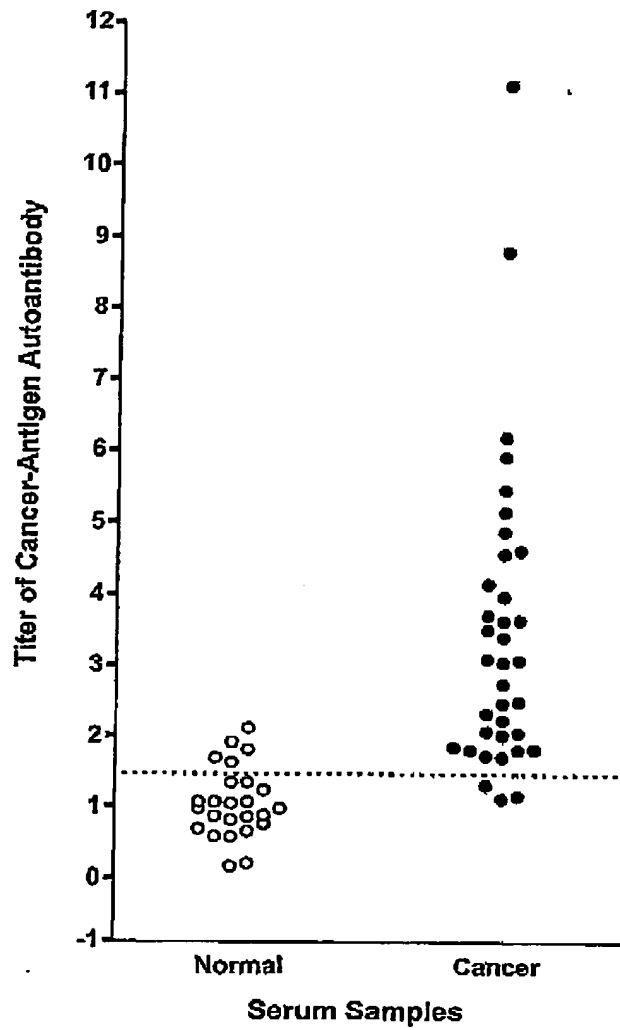


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Figure 6

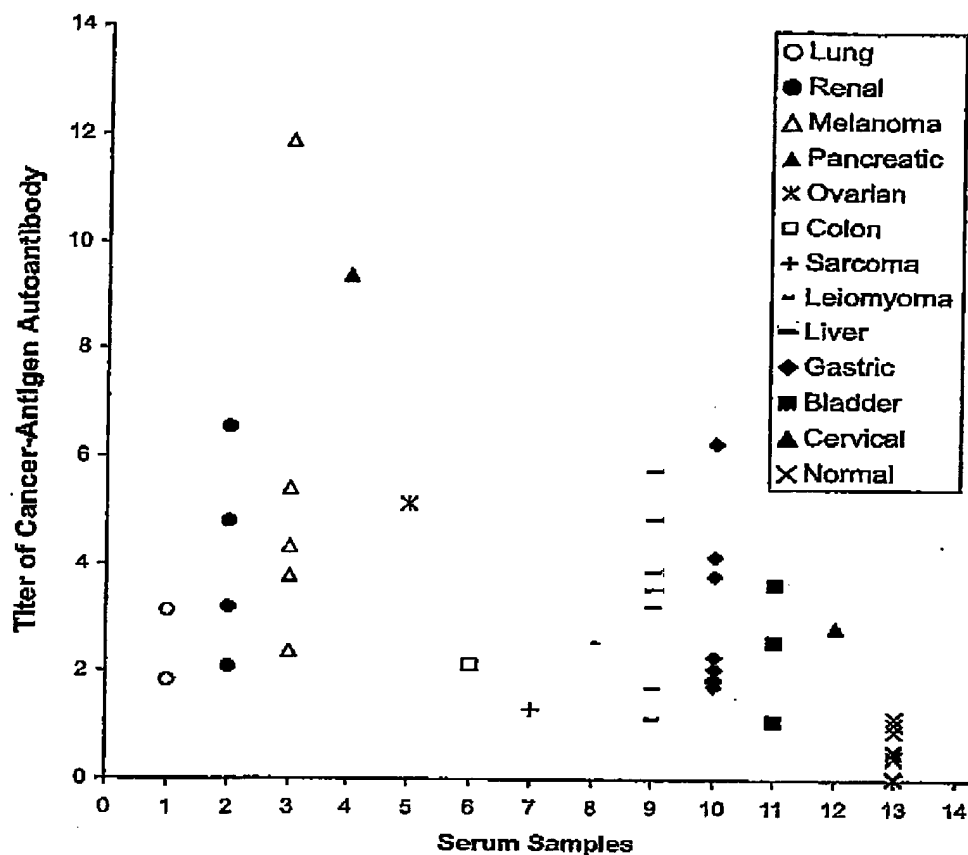


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Figure 7



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US2005/007203

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 GOIN33/574

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 GOIN

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	WO 00/79281 A (THE GOVERNMENT OF THE UNITED STATES OF AMERICA, REPRESENTED BY THE SEC) 28 December 2000 (2000-12-28) abstract; claim 16	1-19
A	CHO Y S ET AL: "Biochemical characterization of extracellular CAMP-dependent protein kinase as a tumor marker." BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS. 30 NOV 2000, vol. 278, no. 3, 30 November 2000 (2000-11-30), pages 679-684, XP009051698 ISSN: 0006-291X page 683, column 1, paragraph 2 abstract	1-19

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

10 August 2005

Date of mailing of the international search report

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European Patent Office, P.B. 5918 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel (+31-70) 340-2040, Tx. 31 651 epo nl

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## INTERNATIONAL SEARCH REPORT

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHO Y S ET AL: "Extracellular protein kinase A as a cancer biomarker: its expression by tumor cells and reversal by a myristate-lacking Calpha and RIibeta subunit overexpression." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA. 18 JAN 2000, vol. 97, no. 2, 18 January 2000 (2000-01-18), pages 835-840, XP009051697 ISSN: 0027-8424 cited in the application page 836, column 1, paragraph 2 abstract	1-19
X	EALLES, LESLEY-JANE: "Immunology for Life Scientists (Second Edition); Chapter 5" 2003, JOHN WILEY AND SONS, LTD , XP002340021 page 176, paragraph 5.2.3.; figure 5.4	10-15
X	JOOS T O ET AL: "A MICROARRAY ENZYME-LINKED IMMUNOSORBENT ASSAY FOR AUTOIMMUNE DIAGNOSTICS" ELECTROPHORESIS, WEINHEIM, DE, vol. 21, 2000, pages 2641-2650, XP001053250 ISSN: 0173-0835 abstract	10-15
A	HONG A, ZHOU Y, DIWU Z: "Phosphopeptides and their biological applications" CHEMISTRY TODAY, June 2003 (2003-06), pages 57-60, XP002339104 the whole document	1-19
P,A	KITA TSUNEKAZU ET AL: "Extracellular cAMP-dependent protein kinase (ECPKA) in melanoma." CANCER LETTERS. 28 MAY 2004, vol. 208, no. 2, 28 May 2004 (2004-05-28), pages 187-191, XP009051699 ISSN: 0304-3835 abstract page 188, column 2, paragraph 2	1-19

## Information on patent family members

International Application No

PCT/US2005/007203

Patent document cited in search report		Publication date		Patent family member(s)	Publication date
WO 0079281	A	28-12-2000	AU	5618700 A	09-01-2001
			WO	0079281 A1	28-12-2000



**Meeting:** 2003 ASCO Annual Meeting  
**Category:** Developmental Therapeutics - Molecular Therapeutics  
**SubCategory:** Gene Therapy/Antisense Strategies



**Gem231, a second-generation anti-PKA oligonucleotide, administered with weekly CPT-11: A phase I trial**

**Abstract No:** 982

**Citation:** Proc Am Soc Clin Oncol 22: page 245, 2003 (abstr 982)

**Author(s):** A. C. Lockhart, C. Rudin, J. Berlin, B. J. Roth, K. R. Hande, R. R. Martin, T. M. Sullivan, J. M. Grindel, R. Zhang, M. L. Rothenberg; Vanderbilt-Ingram Cancer Ctr, Nashville, TN; University of Chicago Medical Center, Chicago, IL; Hybridon, Inc., Cambridge, MA; University of Alabama at Birmingham, Birmingham, AL

**Abstract:** GEM<sup>®</sup>231 is a 2<sup>nd</sup> generation RNA/DNA oligonucleotide targeted at the regulatory RI $\alpha$  subunit of protein kinase A (PKA) I isoform. PKA RI $\alpha$  is upregulated in many cancers and is a primary mediator of cAMP activity. In preclinical models, GEM<sup>®</sup>231 has demonstrated additive or synergistic effects in combination with cytotoxic agents, including CPT-11. This study evaluated the safety, pharmacokinetic, and biological profile of co-administration of CPT-11 (85 mg/m<sup>2</sup> q wk X 4) and GEM<sup>®</sup>231 (40, 80 or 120 mg/m<sup>2</sup>/d for 5d X 4wks) followed by a 2wk rest. CPT-11 and SN-38 plasma concentrations were assessed during wk1 and wk3 of each cycle at 0, 0.5, 1, 2 and 4 hr plus end of infusion. Extracellular PKA (ECPKA) was measured at baseline and weekly thereafter. 11 patients have been enrolled in this ongoing trial. Patient demographics were: 7 male/4 female, median age: 54 (range: 36-71), and median ECOG score: 1 (0:4, 1:6, 2:1). Tumor histologies were colon (4), NSCLC (2), breast (1), prostate (1), ovarian (1), sarcoma (1) and SCLC (1) cancers.

DOSE (CPT-11/GEM-231 mg/m <sup>2</sup> )	# PTS	TOX $\geq$ Gr 3 (DLT: 2 pts* 1 PT#)	MEAN CPT-11 AUC (ng-hr/ml)	MEAN SN-38 AUC (ng-hr/ml)	MEAN $\Delta$ ECPKA (T <sub>1</sub> - T <sub>0</sub> )* (mU/ml)
85/40	4	PS, Fatigue	2973	20.3	-35.6
85/60	2	Neutropenia, leukopenia	NA	NA	NA
85/80	5	PS*, ANC*, Anorexia*, Fatigue*	5590	26.0	-26.4

\*T<sub>0</sub> = baseline; T<sub>1</sub> = after cycle 1.

Due to Grade 4 ANC at the 85/80 mg/m<sup>2</sup> dose, a lower 85/40 mg/m<sup>2</sup> dose level was completed and a 85/60 mg/m<sup>2</sup> dose level was initiated. Preliminary results show that minimum serum ECPKA activity



after GEM<sup>®</sup>231 infusion was significantly ( $p < 0.001$ ) decreased. The CPT-11 AUC was decreased by 28% (wk1 vs. wk3) but the mechanism behind this observation has not been established. SN-38 AUC was not changed (wk1 vs. wk3). The overall SN-38 levels were 80% lower than reported in the literature but this may be artifactual due to early termination of PK sampling. More detailed PK assessment is underway. Evaluation of ECPKA as a biomarker is ongoing. This study was supported by Hybridon and NIH (VICC grant #P30 CA68485).

DOSE (CPT-11/ GEM-231 mg/ m <sup>2</sup> )	# PTS	TOX ≥ Gr 3 (DLT: 2 pts* 1 PT*)	MEAN CPT- 11 AUC (ng- hr/ml)	MEAN SN- 38 AUC (ng-hr/ml)	MEAN Δ ECPKA (T <sub>1</sub> - T <sub>0</sub> ) <sup>*</sup> (mU/ml)
85/40	4	PS, Fatigue	2973	20.3	-35.6
85/80	2	Neutropenia, leukopenia	NA	NA	NA
85/80	5	PS*, ANC*, Anorexia*, Fatigue*	5590	26.0	-26.4

\*T<sub>0</sub> = baseline; T<sub>1</sub> = after cycle 1.

#### Associated Presentation(s):

A presentation was not made on this abstract

#### Other Abstracts in this Sub-Category:

1. A biodistribution study of adenovirus-delivered nitroreductase (Ad-NTR) in patients with operable prostate cancer  
Meeting: 2003 ASCO Annual Meeting Abstract No: 818 First Author: N. D. James
2. A new gene therapeutic strategy using adenovirus dominant negative mutant of C-Jun for colon cancer  
Meeting: 2003 ASCO Annual Meeting Abstract No: 892 First Author: R. Suto
3. A Phase I/II trial of adenoviral p53 gene therapy (INGN 201) for esophageal squamous cell carcinoma.  
Meeting: 2003 ASCO Annual Meeting Abstract No: 877 First Author: T. Ochiai
4. A randomized phase II single center study of gene transfer-based non-specific immunotherapy of malignant mesothelioma (MM) by intratumoral injections of an interleukin-2 producing vero cell line  
Meeting: 2003 ASCO Annual Meeting Abstract No: 920 First Author: J. A. Pitako
5. Ad5CMV-p53 combined with docetaxel (T) and doxorubicin (D) as induction chemotherapy (IC) for patients with locally advanced breast cancer (LABC): Preliminary report of safety and efficacy.  
Meeting: 2003 ASCO Annual Meeting Abstract No: 967 First Author: M. Cristofanilli
6. Adenoviral gene delivery of α(1,3) galactosyltransferase to breast cancer cells: Tumor cell killing by innate immunity  
Meeting: 2003 ASCO Annual Meeting Abstract No: 904 First Author: R. C. Unfer
7. Anti-tumor activity of LY900003 (ISIS3521) in human tumor xenograft models  
Meeting: 2003 ASCO Annual Meeting Abstract No: 861 First Author: X. H. Yang
8. Bcl-2 antisense (G3139, oblimersen) and rituximab enhance chemosensitivity of EBV-associated

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lymphoproliferative disease in vitro and in vivo

Meeting: 2003 ASCO Annual Meeting Abstract No: 863 First Author: J. Lacy

9. Effect of MG98 treatment on DNA gene promoter CpG island methylation patterns in advanced head and neck carcinoma patients

Meeting: 2003 ASCO Annual Meeting Abstract No: 830 First Author: P. C. Amrein

10. Infusion of liposome-encapsulated c-raf antisense oligodeoxynucleotide (LErafAON) during radiation therapy in patients with advanced malignancies: A phase I study

Meeting: 2003 ASCO Annual Meeting Abstract No: 898 First Author: A. Dritschilo

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## Clinical Studies in Patients with Solid Tumors using a Second-Generation Antisense Oligonucleotide (GEM<sup>®</sup>231) Targeted against Protein Kinase A Type I

S. MANI,<sup>a,c</sup> S. GOEL,<sup>a,c</sup> M. NESTEROVA,<sup>d</sup> R.M. MARTIN,<sup>e</sup> J.M. GRINDEL,<sup>f</sup> M.L. ROTHENBERG,<sup>g</sup> R. ZHANG,<sup>h</sup> G. TORTORA,<sup>i</sup> AND Y.S. CHO-CHUNG<sup>d</sup>

<sup>a</sup>Albert Einstein College of Medicine (AECOM), <sup>b</sup>Department of Oncology, and <sup>c</sup>Albert Einstein Comprehensive Cancer Center (AECCC), Bronx, New York 10461, USA

<sup>d</sup>National Cancer Institute, Bethesda, Maryland, USA

<sup>e</sup>Hybridon, Inc., Cambridge, Massachusetts, USA

<sup>f</sup>Pharma Solutions, Alpharetta, Georgia, USA

<sup>g</sup>Vanderbilt Ingram Cancer Center, Nashville, Tennessee, USA

<sup>h</sup>University of Alabama at Birmingham, Birmingham, Alabama, USA

<sup>i</sup>Universita di Napoli Federico II, Napoli, Italy

**ABSTRACT:** GEM<sup>®</sup>231 is a second-generation antisense oligonucleotide targeted against the R1 $\alpha$  regulatory subunit of cAMP-dependent protein kinase type I (PKA-I). Excessive expression of PKA-I is associated with cell proliferation and transformation, and increased levels of secreted extracellular PKA (ECPKA) are found in the serum of cancer patients. Preclinical studies have demonstrated single-agent antitumor activity of GEM 231 in a variety of human cancer xenograft models, and additive or synergistic antitumor activity has been observed with taxane and/or camptothecin-based combinations. Based on prior safety (MTD) data demonstrating dose-dependent, reversible, and cumulative transaminitis, and high peak plasma concentration ( $C_{max}$ )-dependent changes in activated partial thromboplastin time (aPTT) with GEM 231 2-h twice-weekly infusions, an alternative schedule of GEM 231 given as a single agent was evaluated in patients with advanced solid tumors. Fourteen patients (median age ~ 60 yrs) with advanced solid malignancies received a total of 78 weeks of therapy. GEM 231 was infused via a CADD pump at 80 mg/m<sup>2</sup>/day (d) for 3 d/wk (n = 1), then for 5 d/wk at 80 (n = 3), 120 (n = 8), and 180 mg/m<sup>2</sup>/d (n = 2). One cycle was defined as 4 weeks of therapy. Apparent dose dependency for the occurrence of transaminitis was readily reversible. At 180 mg/m<sup>2</sup>/d, 2 of 2 patients had cycle 1 dose-limiting toxicity (DLT) transaminitis. One patient treated at 120 mg/m<sup>2</sup>/d experienced grade 3 transaminase elevations after 8 weeks of therapy, but when serum transaminase values rapidly improved he resumed treatment at 80 mg/m<sup>2</sup>/d for 6 weeks until tumor progression was documented. Another patient at 120 mg/m<sup>2</sup>/d developed grade 3 esophagitis after

Address for correspondence: Sridhar Mani, M.D., Weiler Hospital, Department of Oncology, Rm. 2S-49, 1825 Eastchester Road, Bronx, NY 10461. Voice: 718-904-2629; fax: 718-904-2892. smani@montefiore.org

Ann. N.Y. Acad. Sci. 1002: 252–262 (2003). © 2003 New York Academy of Sciences.  
doi: 10.1196/annals.1281.028

3 weeks, limiting further dosing. One patient (lung cancer) demonstrated stable disease for 9 weeks. Overall, plasma aPTT was minimally prolonged and changes were transient, peaked at the end of each infusion, and were not associated with spontaneous bleeding. A constitutive symptom (e.g., low-grade fatigue) was common, cumulative, and reversible following discontinuation of therapy. Serum ECPKA was measured by enzymatic assay and Western blotting from blood drawn at the beginning and end of each infusion. Serum ECPKA levels demonstrated a trend to decline with the treatment. In addition to single agent schedules, combination trials were undertaken to assess safety and possible interaction of GEM 231 with taxanes (paclitaxel, docetaxel), given once every 3 weeks (one cycle). While trials using the 2-h twice-weekly GEM 231 infusions are ongoing, preliminary results from both studies show that it is safe to combine paclitaxel or docetaxel with GEM 231. Overall, it is also feasible to administer GEM 231 in combination with taxane or nontaxane chemotherapy (e.g., camptothecins). Phase I combination studies are currently underway to further explore the clinical, pharmacokinetic, and biologic profile of GEM 231 with chemotherapy.

**KEYWORDS:** oligonucleotide; PKA type I; solid tumors; antisense; mixed backbone

## INTRODUCTION

Since the introduction of the first antisense drug (fomivirsen) approved by the U.S. Food and Drug Administration for treating CMV-induced retinitis in AIDS patients, the repertoire of antisense oligonucleotides (ASOs) in clinical development has expanded.<sup>1,2</sup> ASOs are relatively devoid of classical chemotherapy-associated side effects; however, there are dose-dependent side effects that require careful vigilance of patients on therapy. These side effects include febrile flu-like symptoms that clinically resemble those from induction of cytokines (e.g., IL6, TNF- $\alpha$ , etc.), asthenia, and alterations in coagulation parameters (e.g., activated thromboplastin time), secondary to oligonucleotide binding and stabilization of the ternary coagulation complex. Thrombocytopenia, hypotension, and transaminase elevations that suggest liver dysfunction<sup>3-5</sup> have also been observed. By employing slow intravenous infusions, great care has been taken in clinical trials to avoid high plasma concentrations that could result in intravascular activation of the complement cascade. First-generation antisense oligonucleotides displayed all these side effects, which were sequence independent or sequence specific (e.g., immune stimulation resulted from CpG motifs).<sup>3,6</sup> Second-generation oligonucleotides have segments of modified deoxy- or ribonucleotides. These modifications have led to better *in vivo* stability, oral bioavailability, and a better concentration-dependent side-effect profile than those of first-generation oligonucleotides.<sup>3-7</sup>

GEM<sup>®</sup>231 (HYB0165, Hybridon, Inc., Cambridge, MA) is an 18-mer antisense oligonucleotide targeted against the human RI $\alpha$  (codons 8-13) regulatory subunit of cAMP-dependent protein kinase type I (PKA-I).<sup>8</sup> This oligonucleotide is a second-generation mixed-backbone construct containing phosphorothioate oligodeoxynucleotide (DNA) with 2'-*O*-methyl ribonucleoside (RNA) modification at the 5' and 3' terminus (Fig. 1). This paper highlights salient clinical features of GEM 231 antisense therapy. Details of each trial mentioned have been submitted for publication elsewhere.

### 5'- GCG UGC CTC CTC AC U GGC -3'

FIGURE 1. GEM<sup>®</sup>231: PKA RI $\alpha$  antisense. 18-mer mixed backbone (RNA-DNA) phosphorothioate oligonucleotide. The underlined bases represent the 2'-O-methyl oligoribonucleosides.

#### PKA IS A VALIDATED TARGET FOR ASO THERAPEUTICS IN CANCER PATIENTS

The PKA holoenzyme is composed of the genetically distinct catalytic (C) and regulatory (R) subunits.<sup>10</sup> They form tetrameric holoenzyme R<sub>2</sub>C<sub>2</sub> that dissociates in the presence of cAMP into an R<sub>2</sub>(cAMP)<sub>2</sub> dimer and two free catalytically active C subunits. The best-known function of the R subunit is inhibition of C subunit kinase activity.<sup>11</sup> Two types of PKA—PKA-I and PKA-II—share a common C subunit but contain distinct R subunits, RI and RII, respectively.<sup>12</sup> Preclinical cancer models *in vitro* and *in vivo* have established PKA-I as a potential therapeutic target.<sup>13–15</sup> Breast cancers, for example, overexpress PKA-I. The RI:RII ratios are significantly higher in normal breast specimens that demonstrate increased proliferation. Patients with high RI:RII ratios have poor prognosis in terms of early disease recurrence and death following primary treatment.<sup>16</sup> Finally, there is a decrease in RI $\alpha$  mRNA following treatment with the antiestrogen tamoxifen in responding but not in nonresponding tumors.<sup>17</sup> In animal model systems of human breast cancers, exposure to antisense RI $\alpha$  oligonucleotides slows cell proliferation and tumor growth.<sup>18</sup> In addition, GEM 231 has demonstrated antitumor activity in a variety of *in vitro* and *in vivo* human tumor models (refs. 18–23; FIG. 2). PKA-I inhibitors (including antisense oligonucleotides) enhance the effects of cytotoxic drugs *in vitro* and *in vivo*.<sup>15</sup> In particular, combination therapy with antiestrogen, camptothecin, taxanes and irinotecan have shown synergy and tolerability.<sup>15–17, 21–23</sup>

#### CLINICAL STUDIES WITH GEM 231

The basis for the first human phase I study<sup>24</sup> using a 2-h infusion administered twice weekly came from the demonstration of efficacy with minimal toxicity in preclinical tumor models. In the published phase I study<sup>24</sup> (Hyb #231-001), seven GEM 231 dose levels were studied using a modified version of the NCI accelerated titration scheme. Fourteen patients (13 evaluable for safety) were enrolled on this study at the two highest dose levels studied; all patients received at least three consecutive doses of GEM 231 infusions (TABLE 1). Toxicity information and detailed parent drug pharmacokinetics were obtained. Pharmacokinetic-pharmacodynamic correlations were performed and, as expected, important observations regarding second-generation ASOs were described. First, there was a linear relationship between high peak plasma concentrations (C<sub>max</sub>) and dose normalized to BSA (r<sup>2</sup> = 0.9218) and dose normalized to body weight in kilograms (r<sup>2</sup> = 0.7753). Similarly, the area under the concentration curve (AUC) increased linearly with dose over an

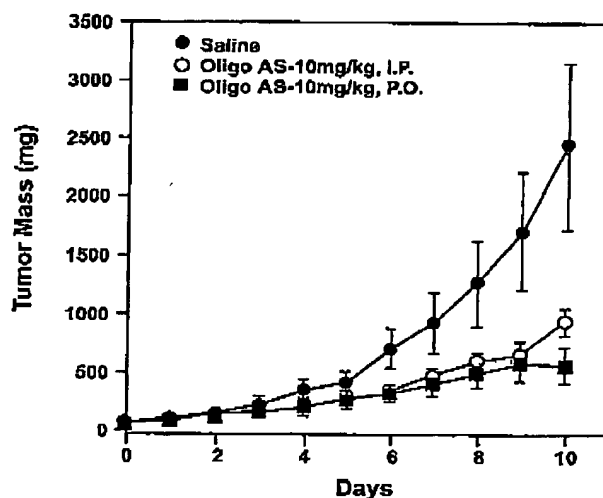


FIGURE 2. Antitumor effect of GEM<sup>®</sup>231 on the growth of the LS174T xenografts in nude mice. GEM231 (10 mg/kg/day) was administered via ip (○) or gavage (■), and saline (●) by ip. Each point represents the mean  $\pm$  SE of tumor mass from six mice.

TABLE 1. Dose escalation and dose-limiting toxicities in cycle 1

Dose Level	Dose (mg/m <sup>2</sup> /d)	N	Weeks of Therapy	DLTs
1	80 $\times$ 3 days	1	3	none
2	80 $\times$ 5 days	3	18	grade 3 pulmonary hypertension
3	120 $\times$ 5 days	8	49	none
4	180 $\times$ 5 days	2	8	grade 3 transaminitis grade 4 transaminitis

LEGEND: DLT = Dose-limiting toxicity; N = number of patients.

18-fold dose range. There was no accumulation of parent drug in plasma with repeated dosing. The plasma half-life of GEM 231 was 0.8–1.6 hours; predicted tissue half-life is likely to be much longer.<sup>6,18</sup> Furthermore,  $C_{max}$  correlated with larger changes in activated partial thromboplastin time (aPTT) from baseline.<sup>6</sup> Preclinical ASO studies suggest that prolonged or protracted delivery may result in prolonged target inhibition.<sup>21,23</sup> Second, there was a dose- and time-dependent effect on serum transaminases, resulting in discontinuation of study drug. Serum transaminases returned to near baseline following discontinuation of therapy. Clinically, multiple (> 2) doses of 360 mg/m<sup>2</sup> proved intolerable due to dose-limiting transaminitis (after 4, 6, or 11 doses) and fatigue. A dose of 240 mg/m<sup>2</sup> was tolerable; however, transient low-grade aPTT elevations (100% of patients) and fever and fatigue (33%) were

noted with multiple dosing. No clinically significant episodes of thrombocytopenia or complement activation were noted. Target effect was not confirmed in this study, and, given the rapid development of dose-limiting transaminitis at higher doses studied, intermittent or infusional schedules were recommended for further evaluation. Based on this concept, and to avoid high peak plasma concentrations, we embarked on a phase I clinical study of GEM 231 administered as a continuous infusion.

#### AEECC TRIALS WITH GEM 231

Infusional GEM 231 therapy was initiated through a phase I study conducted at AEECC between January 2000 and May 2002. The study is closed to accrual and the data audited and cleared for presentation. Because a manuscript has been submitted, only a summary of results is presented. Additional preliminary data in evolution includes results of ECPKA (secreted free catalytic subunit of PKA)<sup>25-27</sup> levels in patients dosed with GEM 231. These results are preliminary and have not been published.

Fourteen patients were enrolled and considered eligible for toxicity and response assessment. The median age was 60 years and > 90% had good performance status (0 or 1 as assessed using the Eastern Cooperative Oncology Group scale) and > 80% had prior chemotherapy. The majority of patients had colorectal cancer (43%) or gynecologic malignancies (~43%). Four dose levels were studied using a modified accelerated dose titration scheme for phase I studies.<sup>28</sup> One cycle of therapy was defined as 4 weeks of continuous therapy. At the two highest dose levels studied—120 mg/m<sup>2</sup>/d and 180 mg/m<sup>2</sup>/d—one and two patients, respectively, developed dose-limiting toxicity in the first cycle of therapy. The recommended phase II dose was 120 mg/m<sup>2</sup>/d for 5 days, repeated every 7 days with no scheduled dosing holiday. At this dose level, eight patients have so far received a median (range) of two (1–4) cycles of therapy.

At the recommended phase II dose (RPTD), toxicities were tolerable; only one patient experienced grade 3 serum transaminase elevations after 8 weeks of therapy. Other liver function tests were not affected and transaminases returned almost to pretreatment values by the third week of interrupted therapy. GEM 231 infusions, resumed at a lower dose of 80 mg/m<sup>2</sup>/d, were administered for another 6 weeks. At that time the transaminases did not increase when treatment was discontinued because disease assessment showed evidence of tumor progression. Plasma aPTT changes were transient; they peaked at the end of each weekly infusion and were not associated with spontaneous bleeding. There was a statistically (but not medically) significant difference between mean pre- and postinfusion aPTT measurements ( $m = 2.05$  secs,  $P = 0.029$ ). Overall, the most significant nonhematologic toxicity was elevation in serum transaminases, usually after 4 weeks or more of therapy. There seemed to be a positive correlation between weekly dose and percent change in aspartate and alanine aminotransferase (AST and ALT) from baseline ( $P < 0.05$ ).

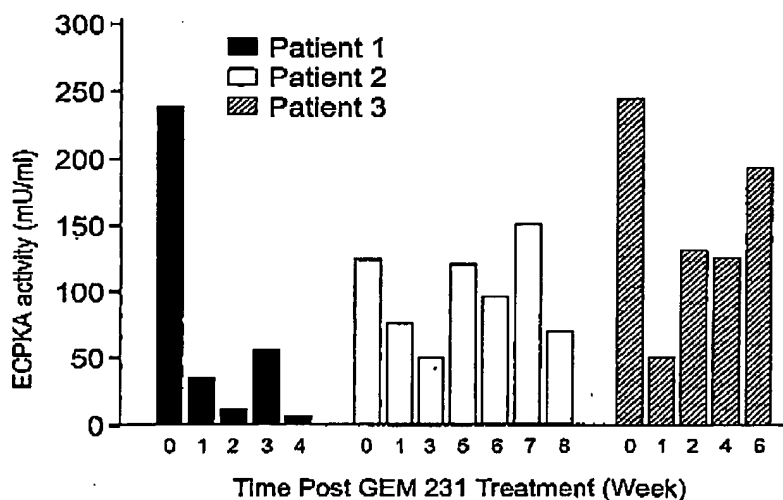
Furthermore, by visual inspection of dose-time-to-onset serum transaminase level plots, the weekly dose seemed to inversely correlate with time to onset of high-grade serum transaminase elevations. Serum transaminase elevations were reversible to near baseline in all patients within 4 weeks of discontinuing therapy. Low-grade fatigue was common, cumulative, and reversible. No objective antitumor responses

were observed; however, one patient (lung cancer) demonstrated stable disease for 9 weeks.

We conclude that GEM 231 is safely administered to cancer patients as a continuous infusion; however, continuous protracted dosing is limited by serum transaminase elevations. Alternative intermittent dosing schedules are likely to improve longer-term tolerability.

During the development of this clinical study, discovery was made of secreted ECPKA in the conditioned medium of cultured cancer cells and the serum of cancer patients.<sup>25-27</sup> ECPKA is present in active, free C subunit form.<sup>25,26</sup> In addition, overexpression of the  $C\alpha$  or  $R1\alpha$  subunit gene of PKA in an expression vector, which upregulates intracellular PKA-I, markedly upregulates ECPKA expression.<sup>25</sup> In contrast, overexpression of the  $R1\beta$  subunit, which eliminates PKA-I, upregulates PKA-II, reverts the transformed phenotype, and downregulates ECPKA.<sup>25</sup> Because downregulation of PKA  $R1\alpha$  results in upregulation of PKA-II ( $R1\beta$ ) in cancer cells,<sup>8,13,14,29</sup> it is postulated that ECPKA could serve as a surrogate to intracellular  $R1\alpha$  depletion.

On the basis of these points, we sought in our study to determine the relationship between GEM 231 dosing and ECPKA. We used enzyme activity and Western blot assay to determine ECPKA expression in patient plasma before and after GEM 231 therapy. While these assays are not yet standardized for clinical use, preliminary data from our trial yielded encouraging results. By Western analysis,  $C\alpha$  expression is detectable in a patient's serum and continued therapy produced a decline in  $C\alpha$  abundance. This assay, however, requires validation and standardization for clinical use.



**FIGURE 3.** Downregulation of ECPKA activity after treatment of cancer patients with GEM<sup>®</sup>231. ECPKA activity was measured by the method previously described<sup>25</sup> in the serum samples from patients before (time zero) and after treatment with GEM 231.



Using the enzymatic assay,<sup>25</sup> ECPKA expression was measured in 14 samples obtained from pretreatment and 56 samples obtained from posttreatment of 14 patients. All serum samples were immediately placed on ice and frozen at  $-80^{\circ}\text{C}$ . Mean ECPKA levels markedly decreased posttreatment with GEM 231. The percentage of patient treatment cycles with decreased ECPKA ranged from 45% to 87% across dose levels with a mean of 55% of all treatment cycles. The mean percent decrease in ECPKA levels ranged from 23.4% to 51.5% with an overall mean of 45.7% (FIG. 3).

Combination phase I studies were recently completed. The combination of GEM 231 with paclitaxel (Taxol) and docetaxel (Taxotere) was based on preclinical laboratory studies showing that taxane antitumor activity against several human tumors was enhanced by the addition of GEM 231.<sup>15</sup> Furthermore, there is a theoretical advantage for downregulation of Rf $\alpha$  because this protein also regulates overexpression of multidrug resistance proteins.<sup>30</sup>

At AECOC, we initiated two trials—one with paclitaxel and the other with docetaxel in combination with GEM 231. In the paclitaxel study (#231-100B), patients with advanced solid tumors were enrolled and treated with one of two paclitaxel and GEM 231 schedules. Schedule A was composed of paclitaxel administered on day 1 as a 3-h infusion followed by GEM 231 twice weekly as a 2-h infusion. Schedule B was composed of paclitaxel administered on day 1 as a 3-h infusion followed by GEM 231 twice weekly for 2 weeks followed by a drug holiday for 1 week. In both schedules, paclitaxel administered once every 3 weeks constituted one cycle of therapy. Because a manuscript has been submitted, only a summary of results is presented.

In schedule A, seven patients were treated across five dose cohorts as shown in TABLE 2. In schedule B, four patients were treated across two dose cohorts. Notably, in schedule A, dose-limiting transaminitis was observed in one patient treated at a  $175\text{ mg/m}^2$  paclitaxel dose and a GEM 231  $220\text{ mg/m}^2$  dose. This dose was not expanded because patients were already entered at the next higher dose level, which consisted of GEM 231 administered at a dose of  $240\text{ mg/m}^2$ . Of four patients entered at the highest dose level, dose-limiting toxicity was not observed; however, one patient with a hepatoma was withdrawn from the study after one dose of the drug. This experience suggests that full doses of both drugs are tolerable for one cycle; with more protracted dosing, however, dose reductions will likely be necessary.

In schedule B, three patients were administered a  $175\text{ mg/m}^2$  paclitaxel dose and a  $240\text{ mg/m}^2$  GEM 231 dose. None of these patients had dose-limiting toxicities and in fact tolerated a longer course of therapy than did similarly dosed patients in schedule A. One patient who was administered a higher dose of GEM 231 at  $360\text{ mg/m}^2$  developed neutropenia after two doses and was removed from the study. Further exploration at this dose was deemed unnecessary because it was demonstrated that the recommended phase II GEM 231 dose could be safely administered on an intermittent basis with full paclitaxel doses.

In the docetaxel study (#231-100A), patients with advanced solid tumors were enrolled and treated with one of two docetaxel and GEM 231 schedules. Schedule A was composed of docetaxel administered on day 1 as a 1-h infusion followed by GEM 231 twice weekly as a 2-h infusion. Schedule B was composed of docetaxel administered on day 1 as a 1-h infusion followed by GEM 231 twice weekly for 2 weeks followed by a 1-week drug holiday. In both schedules, docetaxel administered once every 3 weeks constituted one cycle of therapy. In schedule A, six patients

TABLE 2. Treatment with paclitaxel/GEM<sup>®</sup>231

Patient	Paclitaxel/ GEM <sup>®</sup> dose (mg/m <sup>2</sup> )	Primary cancer	Treatment duration (weeks)	Reason for termination
<b>Schedule A</b>				
01	140/220	gastric	4.5	disease progression
02	175/220	pancreatic	2.0	disease progression
03	175/240-200	pancreatic	6.0	disease progression
04	175/240	supraglottic	6.5	disease progression
05	175/220	colon	3.5	DLT
06	175/240	hepatoma	0.5	AE/disease progression
07	175/240	esophagus	3.5	disease progression
<b>Schedule B</b>				
08	175/240	breast	2.0	disease progression
09	175/240	head & neck	9.0	disease progression
10	175/360	head & neck	1.0	AE
11	175/240	head & neck	10.0	disease progression

LEGEND: DLT = dose-limiting toxicity; AE = adverse event currently being confirmed; see text for definitions of schedules A & B.

were treated across one dose cohort as shown in TABLE 3. In schedule B, 14 patients were treated across three dose cohorts.

In schedule A, six patients were treated at a 50 mg/m<sup>2</sup> docetaxel dose and a 220 mg/m<sup>2</sup> GEM 231 dose. Two patients had dose-limiting toxicities. One patient developed grade 3 fatigue and neuropathy after the third GEM 231 infusion; the second developed grade 3 transaminase elevation at the end of cycle 1. Because the majority of patients could not complete more than one cycle of treatment (mean duration ~3.2 weeks) largely due to disease progression, the study was altered to include schedule B and less morbid patients, and eligibility criteria required that patients be thoroughly screened for coagulopathies and histories of concurrent illnesses. While patients with stage IV disease were allowed, a priority for accrual was to find patients with good performance status and prior chemotherapy tolerability.

In schedule B, 14 enrolled patients underwent a mean treatment duration of ~3.7, weeks with 100% completing one full treatment cycle and 82% completing two or more treatment cycles. In schedule B, no dose-limiting toxicity was observed at a 50 mg/m<sup>2</sup> docetaxel dose. At a 60 mg/m<sup>2</sup> dose, one of four patients developed grade 3 transaminase elevation on day 4 of treatment that resolved by day 11. Because we did not establish criteria for a maximum tolerated dose, three patients were entered at the next docetaxel/GEM 231-dose level. One patient had a dose-limiting toxicity, which would have resulted in an expansion of the dose to six patients. We have treated four patients at this dose level with no subsequent dose-limiting events. Further patients at this level are warranted to accurately define a safe and tolerable dose.

The Vanderbilt-Ingram Cancer Center, in collaboration with the University of Chicago, has embarked on a phase I study to evaluate the feasibility and safety of irinotecan given in combination with GEM 231.<sup>31</sup> This combination is based on ele-

TABLE 3. Treatment with docetaxel/GEM<sup>®</sup>231

Patient	docetaxel/ GEM <sup>®</sup> dose (mg/m <sup>2</sup> )	Primary cancer	Treatment duration (weeks)	Reason for termination
<b>Schedule A</b>				
01	50/220	rectal	4.0	disease progression
02	50/220	bone	2.0	disease progression
03	50/220	colon	2.5	DLT
04	50/220	head & neck	1.5	AE
05	50/220	unknown	6.0	AE
06	50/220	colon	3.5	disease progression
<b>Schedule B</b>				
07	50/220	colon	2.0	disease progression
08	75/220	pancreatic	4.0	disease progression
09	50/220	head & neck	4.5	disease progression
10	50/220	lung	4.0	disease progression
11	50/220	lung	3.0	disease progression
12	50/220	lung	4.0	disease progression
13	50/220	breast	4.0	disease progression
14	60/220	salivary gland	4.5	disease progression
15	60/220	breast	3.0	disease progression
16	60/220	breast	2.5	AE
17	60/220	pancreatic	4.0	AE
18	75/220	lung	2.0	DLT
19	75/220	uterine	8.0	AE (drug related)
20	75/220	prostate	12.0	disease progression

Legend: DLT = dose-limiting toxicity; AE = adverse event currently being confirmed; see text for definitions of schedules A & B.

gant preclinical studies from Zhang and Agrawal demonstrating synergy of cytotoxic effect with minimal apparent toxicity.<sup>21,23</sup> Furthermore, there is preliminary evidence for modulation of SN-38 and SN-38 glucuronide pharmacokinetics in tissue that may favor enhanced cytotoxic activity (R. Zhang, unpublished data). In the current human cancer study, irinotecan (85 mg/m<sup>2</sup> intravenously every week for 4 consecutive weeks) is administered with GEM 231 (40, 80, or 120 mg/m<sup>2</sup>/d for 5 days each week for 4 consecutive weeks) followed by a 2-week rest. Preliminary results show that minimum serum ECPKA activity is decreased after GEM 231 infusion, but larger sample sizes are needed to confirm this effect. The pharmacokinetic-pharmacodynamic relationships are still too preliminary to characterize or define.<sup>31,32</sup>

In summary, GEM 231 used as a single agent in patients with cancer is well tolerated on 2-hour and continuous infusion schedules. To administer GEM 231 for longer-term use, intermittent infusional schedules may be most appropriate. Clearly, a test using patient serum that could indicate the effect of an anticancer drug on its target would be of great interest.<sup>32-34</sup> Many more patient-based data are required, however, to determine whether the ECPKA test would serve this purpose. Despite

the lack of significant cytotoxic efficacy in these phase I trials, it is encouraging that GEM 231 can be safely administered with taxanes (paclitaxel and docetaxel) and topoisomerase I inhibitors (irinotecan). Trials using these combinations are warranted, as are trials of GEM 231 in combination with irinotecan. The data for the latter combination are still in evolution; however, compelling preclinical data warrant a full-scale development plan with irinotecan.

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